PA 1053163

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

August 15, 2003

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: ***60/400,129***
FILING DATE: ***August 02, 2002***

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. WILLIAMS
Certifying Officer

PTO/SB/16 (10-01)
Approved for use through 10/31/2002. OMB 0651-0032
Patent and Trademark Office, U S DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

Express Mail Label No.

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
JANICE BETH YEVED	RICHMAN-EISENSTAT	Winnipeg, Manitoba, Canada
JING	YU	Winnipeg, Manitoba, Canada

☐ *Additional inventors are being named on the ____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

MODULATION OF AIRWAY SMOOTH MUSCLE CELLS

CORRESPONDENCE ADDRESS

Direct all correspondence to:

☒ Customer Number 1059 → *Place Customer Number Bar Code Label Here* 01059 PATENT & TRADEMARK OFFICE

OR *Type Customer Number here*

☒ Firm *or* Individual Name	BERESKIN & PARR				
Address	40 King Street West				
Address					
City	Toronto	State	Ontario	ZIP	M5H 3Y2
Country	Canada	Telephone	416-364-7311	Fax	416-361-1398

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 49 ☐ CD(s), Number ____

☒ Drawing(s) *Number of Sheets* 15 ☐ Other (specify)

☒ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☒ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order is enclosed to cover the filing fees

FILING FEE AMOUNT ($)

☐ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 022095

☐ Payment by credit card. Form PTO-2038 is attached.

$80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: ____.

Respectfully submitted,

SIGNATURE _____ Date AUG. 1, 2002

TYPED or PRINTED NAME MICHELINE GRAVELLE REGISTRATION NO. *(if appropriate)* 40,261

TELEPHONE 416-364-7311 Docket Number: 9157-26

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1 51 The information is used by the public to file (and by the PTO to process) a provisional application Confidentiality is governed by 35 U S C 122 and 37 CFR 1 14 This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO Time will vary depending upon the individual case Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U S Patent and Trademark Office, U S Department of Commerce, Washington, D C , 20231 DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS SEND TO Box Provisional Application, Assistant Commissioner for Patents, Washington, D C 20231

PTO/SB/17 (11-01)
Approved for use through 10/31/2002 OMB 0651-0032
U S Patent and Trademark Office, U.S DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

FEE TRANSMITTAL for FY 2002

Patent fees are subject to annual revision

☑ Applicant claims small entity status See 37 CFR 1 27

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete If Known	
Application Number	
Filing Date	
First Named Inventor	JANICE BETH YEVED RICHMAN-EISENSTAT
Examiner Name	
Group Art Unit	
Attorney Docket No.	9157-26

METHOD OF PAYMENT *(check all that apply)*

☑ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☐ Deposit Account #4534

Deposit Account Number: 022095

Deposit Account Name: Bereskin & Parr

The Commissioner is authorized to: *(check all that apply)*

☐ Charge fee(s) indicated below ☑ Credit any overpayments

☐ Charge any additional fee(s) during the pendency of this application

☐ Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims		Fee from below	Fee Paid
Total Claims	- 20** =		X		= 0.00
Independent Claims	- 3** =		X		= 0.00
Multiple Dependent					=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent claim, if not paid
109	84	209	42	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0.00

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for *ex parte* reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	400	216	200	Extension for reply within second month	
117	920	217	460	Extension for reply within third month	
118	1,440	218	720	Extension for reply within fourth month	
128	1,960	228	980	Extension for reply within fifth month	
119	320	219	160	Notice of Appeal	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,280	241	640	Petition to revive - unintentional	
142	1,280	242	640	Utility issue fee (or reissue)	
143	460	243	230	Design issue fee	
144	620	244	310	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Processing fee under 37 CFR 1 17(q)	
126	180	126	180	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	740	246	370	Filing a submission after final rejection (37 CFR § 1 129(a))	
149	740	249	370	For each additional invention to be examined (37 CFR § 1 129(b))	
179	740	279	370	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 0.00

SUBMITTED BY				Complete *(if applicable)*	
Name *(Print/Type)*	Micheline Gravelle	Registration No *(Attorney/Agent)*	40,261	Telephone	(416) 364-7311
Signature	M. Gravelle			Date	AUGUST 1, 2002

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

Burden Hour Statement: This form is estimated to take 0 2 hours to complete Time will vary depending upon the needs of the individual case Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U S Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO. Assistant Commissioner for Patents, Washington, DC 20231.

Petition included?::	No
Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers::	
Secrecy Order in Parent Appl.?::	No

Applicant Information

Inventor Authority Type::	Inventor
Primary Citizenship Country::	Canada
Status::	Full Capacity
Given Name::	JANICE
Middle Name::	BETH YEVED
Family Name::	RICHMAN-EISENSTAT
Name Suffix::	
City of Residence::	Winnipeg
State or Prov. Of Residence::	Manitoba
Country of Residence::	CANADA
Street of mailing address::	195 Montrose St.
City of mailing address::	Winnipeg
State or Province of mailing address::	Manitoba
Country of mailing address::	CANADA

[redacted]

Postal or Zip Code of mailing address::	R3M 3L9
Inventor Authority Type::	Inventor
Primary Citizenship Country::	China
Status::	Full Capacity
Given Name::	JING
Middle Name::	
Family Name::	YU
Name Suffix::	
City of Residence::	Winnipeg
State or Prov. Of Residence::	Manitoba
Country of Residence::	CANADA
Street of mailing address::	798 McDermot Ave., Suite #2
City of mailing address::	Winnipeg
State or Province of mailing address::	Manitoba
Country of mailing address::	CANADA
Postal or Zip Code of mailing address::	R3E 0T5

Correspondence Information

Correspondence Customer Number::	001059
Phone Number::	(416) 364-7311
Fax Number::	(416) 361-1398

Initial 8/1/02

E-Mail Address:: mgravelle@bereskinparr.com

Representative Information

Representative Customer Number:: 001059

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::

Foreign Priority Applications

Country::	Application Number::	Filing Date::	Priority Claimed

Assignee Information

Assignee name::

Street of mailing address::

City of mailing address::

State or Province of mailing address::

Country of mailing address::

Postal or Zip Code of mailing address::

[redacted]

Patent Application Data Sheet

Application Information

Application Type::	Provisional
Subject Matter::	Utility
Suggested Classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Number of CD disks::	0
Number of copies of CDs::	0
Sequence submission?::	NO
Computer Readable Form (CRF)?::	NO
Number of copies of CRF::	0
Title::	MODULATION OF AIRWAY SMOOTH MUSCLE CELLS
Attorney Docket Number::	9157-26
Request for Early Publication?::	NO
Request for Non-Publication?::	NO
Suggested Drawing Figure::	3
Total Drawing Sheets::	15
Small Entity?::	Yes
Latin Name::	
Variety denomination name::	

B&P File No. 9157-26

BERESKIN & PARR **UNITED STATES PROVISIONAL**

Title: MODULATION OF AIRWAY SMOOTH MUSCLE CELLS

Inventor: JANICE BETH YEVED RICHMAN-EISENSTAT and JING YU

B&P File No. 9157-26

TITLE: MODULATION OF AIRWAY SMOOTH MUSCLE CELLS

FIELD OF THE INVENTION

The invention relates to methods of modulating intracellular calcium signalling in airway smooth muscle cells, methods of treating asthma and methods of drug delivery to air way smooth muscle cells, as well as methods to diagnose IgA-receptor-mediated bronchial hyperreactivity.

BACKGROUND OF THE INVENTION

Asthma is characterized by bronchial hyperreactivity to a variety of stimuli. A number of studies indicate a major role for alterations in the smooth muscle. Airway smooth muscle (ASM) cells exhibit a contractile phenotype and a proliferative-synthetic phenotype, capable of producing proinflammatory cytokines, chemokines and growth factors (Halayko et al, 1996; Halayko et al, 1999; Schmidt and Rabe, 2000). It is now being suggested that the ASM itself can contribute directly to the persistence of inflammation and airway remodeling that occurs in asthma. The features of this airway remodeling include epithelial damage, deposition of extracellular matrix proteins throughout the airways, goblet cell metaplasia, and smooth muscle hypertrophy (Holgate et al, 2000). It is unknown whether this remodeling is due to or occurs in parallel with the inflammatory response.

Little is known about the biology of IgA in asthma. However, upper airway infections are well known to frequently exacerbate the airflow obstruction that occurs in patients with asthma – a situation where the concentration of immunoglobulins in the airways increases. If ASM indeed possess receptors for IgA that alter ASM biology or function, then a new pathophysiological explanation arises for these infectious exacerbations of asthma. In addition, the presence or absence of such a receptor on ASM might account, at least in part, for the variety of clinical manifestations and therapeutic responsiveness amongst patients with asthma. Furthermore, a better understanding of the cell and molecular biology of such receptors and

their biology in ASM could lead to a novel approach for the diagnosis and treatment of asthma.

Immunoglobulin A (IgA) abundantly coats the enormous surface area of the mucosal epithelium, which measures about 300-400 m^2 in adult humans. Despite its abundance, relatively little is known about the mechanism of action of IgA in host immune defense and immune tolerance. The mucosal epithelium of the upper airway functions as a critical barrier that must protect the internal environment from potential pathogens and toxins in the external environment.

The mucosal epithelium is physically vulnerable to continuous exposure to potentially infectious agents, such as bacteria, viruses, fungi and parasites, as well as to substances in the environment or diet. IgA is one of the most important proteins protecting the mucosal epithelium that guards the internal environment from the outside world. Elevated concentrations of IgA have been identified in induced sputum from asthmatics in contrast to that from healthy people (Louis et al, 1997; Nahm and Park, 1997). Furthermore, increased levels of specific IgA antibodies to both allergen and bacterial antigen have been measured in induced sputum from asthmatics (Nahm et al, 1998).

Polymeric Immunoglobulin Receptor

IgA exists in different isoforms (Mestecky et al, 1999). B lymphocytes residing in submucosal tissues produce similar proportions of polymeric IgA1 and IgA2 subclasses, secreting at least two IgA molecules linked together by a J chain. Epithelial cells of the respiratory and gastrointestinal tracts abundantly express the polymeric immunoglobulin receptor (pIgR) which serves to transcytose polymeric IgA from the submucosa (the basolateral surface of the epithelium) to the lumenal (apical) surface. At the apical surface, proteolytic cleavage of the pIgR releases secretory component (SC) bound to dIgA into mucosal secretions, called secretory IgA (sIgA). SC stabilizes sIgA from proteolytic degradation by bacterial enzymes and helps neutralize pathogens, especially viruses. sIgA in mucosal secretions is the first line of defense, acting to bind microorganisms and thereby limiting

adhesion and colonization. IgA may neutralize viruses and bacterial toxins by binding to antigenic determinants important in the microorganism's interaction with cellular receptors. Additional roles for sIgA are postulated to include transport of immune complexes out through the epithelial surface by the pIgR.

In contrast to mucosal secretions where sIgA prevails, the predominant form of IgA in human serum is monomeric IgA (mIgA) from B lymphocytes in the bone marrow and spleen. While the pIgR will selectively mediate transport of polymeric IgA across epithelial cells, this receptor does not bind monomeric IgA. IgA present in secretions therefore differs in biochemical properties from IgA found in serum. The polymerization state and the presence of SC might be expected to result in unique effector functions for different forms of IgA depending on the site of production and intended point of action.

Mucosal epithelial cells of the airway and intestine abundantly express the pIgR which functions to transfer its ligand, polymeric IgA (pIgA), from the blood and submucosal B lymphocytes (basolateral surface) to the lumenal (apical) surface and into external secretions (Mostov et al, 1995). IgA is one of the first lines of host immune defense in mucosal secretions. The lung is the second greatest site of dIgA transport, exceeded only by the intestine. Total IgA transport is roughly 5-15 gms per day in an adult human with 15% transported into airway secretions, so pIgR transcytosis is clearly a significant pathway (Childers et al, 1989). In fact, IgA represents about 5-10% of the total protein in bronchoalveolar lavage fluid (Bell et al, 1981). The pIgR and bound ligands are very rapidly endocytosed from the basolateral surface of the epithelial cells, delivered to endosomes, and eventually transcytosed via vesicles to the apical surface of the epithelial cell. At the apical surface, the extracellular, ligand-binding domain of the pIgR is proteolytically cleaved and released together with its ligand into external secretions. This cleaved fragment of the pIgR is known as secretory component (SC). SC bound to polymeric IgA is known as secretory IgA (sIgA), and stabilizes IgA against proteolytic degradation by bacterial enzymes.

Cleavage of the pIgR at the apical surface is not extremely rapid, so there is a pool of uncleaved pIgR at the apical surface. Most of the apically

endocytosed ligand recycles back to the apical surface. In contrast, only 20% of pIgR at the basolateral surface recycles back to the basolateral surface. The predominant route is for 80% of pIgR to traffic from the basolateral to the apical surface. These trafficking events occur regardless of whether pIgR is bound to its ligand, dIgA. The molecular determinants of these protein trafficking events are encoded in the 103 amino acid cytoplasmic domain of pIgR (Mostov et al, 1995). This domain contains highly conserved signals for intracellular sorting and transcytosis, including signals for rapid endocytosis and for avoiding degradation in lysosomes. Upon binding of pIgR to pIgA, phospholipase C is activated and phosphokinase C stimulate apical delivery, IP3 releases calcium and calmodulin sequesters the basolateral retrieval signal. Gsα, cAMP and protein kinase A stimulate apical delivery of pIgR (Mostov and Kaetzel, 1999). The trafficking events of pIgR across epithelial cells are clearly under very tight control.

pIgA binds to the pIgR on mucosal epithelial cells via the J chain. In addition, pIgA, which is heavily glycosylated, can bind to asialoglycoprotein receptors on liver cells. In contrast, binding to white blood cells (neutrophils, eosinophils, monocytes/macrophages) occurs by attachment of the Fc portion of IgA to FcαR (also known as CD89) expressed on these cells (Kerr and Woof, 1999; Morton et al, 1996).

Fc-alpha Receptors For IgA

Binding to white blood cells (neutrophils, eosinophils, monocytes/macrophages) occurs by attachment of the Fc portion of IgA to Fc-alpha receptors (FcαR; also known as CD89) expressed on these cells (Kerr and Woof, 1999; Morton et al, 1996). Neutrophils and monocytes/macrophages constitutively express FcαR as a 55-75 kd protein, while eosinophils express FcαR as a 70-100 kd protein with increased glycosylation (Albrechtsen M, et al, 1988; Monteiro et al, 1990). FcαR expression on monocytes and neutrophils increases in response to TNF-α, IL-1, GM-CSF, LPS or phorbol esters; IFN-γ and TGF-β1 decrease expression (discussed in Deo et al, 1998). The gene for FcαR is located on chromosome

19 and encodes several alternatively spliced isoforms of the receptor's α-chain (55-110 kD; Morton et al, 1996). FcαR can trigger release of inflammatory mediators and phagocytosis of IgA-coated particles (Yeaman and Kerr, 1987; Patry et al, 1995). IgA-coated neutrophils and macrophages phagocytose particles, bacteria and immune complexes more efficiently than uncoated cells. Although the concentration of the predominantly monomeric IgA in blood is high enough to completely saturate the thousands of FcαR on neutrophils, mIgA will not trigger signal transduction in PMNs unless the receptors are crosslinked (Stewart et al., 1994). The pIgA and sIgA have the potential to crosslink FcαR on cell surfaces due to their polymeric composition. So, during times of infection when submucosal B cells are stimulated to increase production of specific pIgA, myeloid cells recruited to sites of inflammation are better prepared for their functions in the mucosal lumen. FcαR-induced calcium release and subsequent cytokine production depend on association with the FcR γ-chain (Morton et al, 1995). In vivo studies in transgenic mice show that while FcR γ chain is important for FcαR-triggered phagocytosis, CR3 (CD11b/CD18) is required for FcαR-mediated antibody-dependent cellular cytotoxicity (van Egmond et al, 1999).

FcαR may play a role in cancer in addition to its function against microbial pathogens: IgA antitumor antibodies or bispecific antibodies directed to FcαR and tumor antigens effectively lyse tumor cells (Deo et al, 1998). Deo's work and that of others highlight FcαR as a potential immunotherapeutic target of malignant and infectious diseases (Valerius et al, 1997; van de Winkel et al, 1997). The novel finding of the FcαR on ASM thus indicates that targeting this receptor would be a promising and novel therapeutic approach for inflammatory diseases, such as asthma.

Airway smooth muscle

ASM reside within the internal mucosal environment, below the epithelial cells. Infections of the upper airway stimulate submucosal B cells to increase production of specific pIgA. Furthermore, if infectious pathogens resulted in the breakdown of the epithelial barrier, then cells in the

subepithelial layers, such as those of ASM, become exposed to increased concentrations of pIgA as well as sIgA. Although airway inflammation and bronchoconstriction involve multifactorial and complex processes, if ASM possess receptors for IgA that are activated by different isoforms of IgA, then a novel pathophysiological mechanism is proposed to account for (1) the deterioration in airflow obstruction during infectious exacerbations in patients with asthma, (2) the induction of asthma in predisposed individuals, and (3) the temporary development of bronchial hyperreactivity in non-asthmatics (i.e. reactive airways dysfunction syndrome).

Although all asthmatics manifest bronchial hyperreactivity, the stimuli or precipitants of an asthmatic attack or exacerbation can vary from patient to patient. Examples of exacerbating factors for asthma include exercise, cold air, increased humidity, upper airway infection, sinus infection, gastric aspiration or gastroesophageal reflux, a variety of allergens (e.g. house dust mite, pollens, grass), fumes (e.g. cigarette smoke, paint fumes, dusts). Not all asthmatics are alike in their responses to these different stimuli. Some people react immediately to a stimulus; others have delayed bronchial constriction. Furthermore, different patients respond differently to currently available medications for asthma. Expression of IgA receptors on ASM could account for these differences amongst asthmatics and non-asthmatics with bronchial hyperreactivity, and therefore comprise a novel diagnostic tool.

SUMMARY OF THE INVENTION

The inventors have unexpectedly found a protein similar to the polymeric immunoglobulin receptor (pIgR) expressed in human airway smooth muscle (ASM) cell cultures. The inventors have also shown that incubation of ASM with the ligand for pIgR (pIgA) causes a rise in intracellular calcium concentrations that is unique in that the response is delayed, sustained, oscillates and increases the sensitivity of ASM to subsequent stimulation with histamine. Incubation with mIgA (which does not bind pIgR) does not cause this effect on intracellular calcium concentrations in ASM. Smooth muscle responses to IgA have never been described before.

Furthermore, the inventors have unexpectedly found that human ASM express FcαR when cultured in the presence of serum. This expression is increased when ASM cells are pre-incubated with either mIgA or pIgA, both of which can bind the FcαR.

Accordingly, the present invention provides a method of modulating intracellular calcium signalling in an airway smooth muscle cell comprising administering to a cell or animal in need thereof an effective amount of an agent that can modulate an IgA receptor, such as pIgR or FcαR on an airway smooth muscle cell.

In one embodiment, the present invention provides a method of treating a patient with asthma comprising administering an effective amount of an IgA receptor antagonist to a patient in need thereof.

The discovery of the pIgR and FcαR on airway smooth muscle cells allows the development of methods to target delivery of a compound or substance to an airway smooth muscle cell. Accordingly, the present invention also includes a method of delivering a substance to a mesenchymal cell comprising administering an effective amount of a conjugate comprising the substance coupled to an IgA receptor ligand to an animal or cell in need thereof.

The discovery of the pIgR and FcαR on airway smooth muscle cells also allows the development of diagnostic assays to detect IgA receptor mediated diseases such as asthma.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1A shows immunofluorescence staining for pIgR protein in serum-starved ASM using a rabbit antibody to human secretory component.

Figure 1B shows immunofluorescence staining for the alpha chain of IgA in serum-starved ASM pre-incubated overnight with pIgA (live-cell uptake) in contrast to pre-incubation with mIgA.

Figure 1C shows live uptake staining for the rabbit IgG antibody to human secretory component in contrast to pre-incubation of live ASM cells with an irrelevant rabbit IgG.

Figure 2 shows immunofluorescence staining for FcαR in non-starving ASM cells (upper panel) and that this staining is enhanced by pre-incubating live cells with either pIgA (middle panel) or mIgA (lower panel).

Figure 3 shows a western blot for pIgR protein in non-starving ASM that is still present after 6 days and 12 days of serum starvation (upper panel). This blot was stripped and re-probed with a phosphotyrosine antibody to show that the pIgR protein in ASM is less phosphorylated with serum deprivation.

Figure 4 shows a pIgR band in serum-starved ASM by RT-PCR. MDCK cells transfected with pIgR were used as a positive control.

Figure 5 shows the presence of FcαR mRNA in non-starving ASM and U937 positive control cells in contrast to serum-starved ASM.

Figure 6A shows calcium imaging studies in Fura-2 loaded serum-starved ASM stimulated with 12 μg/ml pIgA. At 80 min, pIgA causes an initial rise in intracellular calcium concentrations and this effect becomes sustained at 110 min. At this point, the calcium concentrations oscillate. The oscillations abate and concentrations decrease 10 min after washing off the pIgA.

Figure 6B shows calcium imaging studies in Fura-2 loaded serum-starved ASM in buffer alone (time course study).

Figure 7A shows calcium imaging studies in Fura-2 loaded serum-starved ASM pre-stimulated with histamine which is washed off prior to adding low dose pIgA (0.12 μg/ml). Intracellular calcium concentrations begin to rise after 1 h and increase 4-6 fold with histamine stimulation after washing off pIgA.

Figure 7B shows calcium imaging studies in Fura-2 loaded serum-starved ASM incubated with buffer alone (time control) after pre-stimulation with histamine. These cells were re-exposed to histamine after 2 hours in buffer and showed less than a 2-fold increase in intracellular calcium concentrations.

Figure 7C shows calcium imaging studies in Fura-2 loaded serum-starved ASM stimulated with mIgA following pre-stimulation with histamine. (These serum-starved ASM do not express FcαR making mIgA a negative control.) After washing off mIgA, the responses to histamine are augmented by a factor of less than 2, as in Figure 7B with buffer alone.

Figure 7D shows calcium imaging studies in Fura-2 loaded serum-starved ASM stimulated with high dose pIgA 12µg/ml following stimulation with histamine as well as carbachol. Intracellular calcium concentrations begin to rise 50 min later and go off-scale at 66 min with over a 30-fold increase.

Figure 8 shows a series of images obtained during Fura-2 calcium imaging studies with serum-starved ASM stimulated with 12 µg/ml pIgA. The cell that is circled in Frame 1 contracts and disappears by Frame 11.

Figure 9 shows a western blot confirming that the different scFv clones recognize purified J-chain protein.

DETAILED DESCRIPTION OF THE INVENTION

I. Therapeutic Methods

As mentioned above, the present inventors have determined that IgA receptors, including pIgR and Fc-alpha R, are present on ASM cells and that binding the receptor causes calcium signalling. Therefore the present invention includes all diagnostic and therapeutic methods for treating conditions that are mediated through modulation of calcium signalling through IgA receptors on airway smooth muscle cells.

Broadly stated, the present invention provides a method of modulating intracellular calcium signalling in an airway smooth muscle cell comprising administering to a cell or animal in need thereof an effective amount of an agent that can modulate an IgA receptor on an airway smooth muscle cell.

The term "modulate" as used herein includes the inhibition or suppression of a function or activity as well as the induction or enhancement of a function or activity and interference with the interaction between any isoform of IgA and its receptor such as pIgR or FcαR. For example, an agent that can modulate IgA receptors includes agents that can inhibit or block the signalling through an IgA receptor (receptor antagonists) as well as agents that can induce or stimulate signalling through an IgA receptor (receptor agonists).

The term "IgA receptor" means any receptor on an airway smooth muscle cell that can bind any isoform of IgA. The receptor may also bind other immunoglobulins. In a preferred embodiment, the IgA receptor on the airway smooth muscle cell is pIgR or FcαR.

The term "pIgR" as used herein denotes a polymeric immunoglobulin receptor and means a receptor on cells that binds polymeric IgA (pIgA), dimeric IgA (dIgA) and polymeric IgM (pIgM) but not monomeric forms of IgA. The term includes the pIgR that has been previously described on epithelial cells (Piskurich et al., *J. Immuno.* 154:1735-1747, 1995) as well as any analogs, homologues, derivatives or variants (including splice variants) of the known pIgR molecules.

The term "FcαR" as used herein denotes the Fc-alpha receptor, also known as CD89, and means a receptor on cells that binds any isoform of IgA by its Fc portion. The term includes the FcαR that has been previously described on white blood cells (Morton et al, *Crit. Rev. Immunol.* 16: 423-440, 1996) as well as any analogs, homologues, derivatives or variants (including splice variants) of the known FcαR molecules.

The term "a cell" as used herein includes a single cell as well as a plurality or population of cells. Administering an agent to a cell includes both *in vitro* and *in vivo* administrations.

The term "animal" as used herein includes all members of the animal kingdom, including humans. Preferably, the animal to be treated is a human.

The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired result, e.g. to modulate calcium signalling.

In one embodiment, the present invention provides a method of preventing or inhibiting intracellular calcium signalling in an airway smooth muscle cell comprising administering an effective amount of an IgA receptor antagonist to a cell or animal in need thereof.

The term "preventing or inhibiting intracellular calcium signalling" means that the intracellular level of calcium in an airway smooth muscle cell in the presence of the IgA receptor antagonist is decreased as compared to the level of intracellular calcium in cells in the absence of the agent. Calcium levels can be measured using a variety of known techniques including using fluorescence spectrophotometric and imaging techniques as described in Example 1.

The IgA receptor antagonist can be any agent that inhibits signalling through an IgA receptor and results in an inhibition of intracellular calcium signalling or an inhibition of IgA receptor-mediated endocytosis.

In a preferred embodiment, the IgA receptor antagonist will inhibit the binding of pIgA to pIgR or FcαR on airway smooth muscle cells by either binding to the portion of the IgA molecule that interacts with the IgA receptor or by binding to the portion of the IgA receptor that binds the IgA molecule The IgA receptor antagonist may be an antibody that binds, but does not activate the pIgR or FcαR on airway smooth muscle cells, and results in an inhibition of the binding of IgA with the resultant inhibition of intracellular calcium signalling. Other IgA receptor antagonists include anti-J chain antibodies that might interfere with the ability of pIgR to bind pIgA or pIgM or antibodies or ligands to the portion of the Fc-alpha part of IgA that binds to Fc-alpha receptor on airway smooth muscle cells. Examples of other IgA receptor antagonists are provided in Section II.

Intracellular calcium signalling is important for several processes in cell biology, including cell division, cytokine/chemokine/growth factor production, cell movement and contraction. Therefore, inhibiting calcium signalling can

inhibit a variety of calcium dependent effects. Accordingly, the present invention provides a method of inhibiting the contraction of an airway smooth muscle cell comprising administering an effective amount of an IgA receptor antagonist to a cell or animal in need thereof.

The present invention further provides a method of inhibiting the production of inflammatory mediators or growth factors comprising administering an effective amount of an IgA receptor antagonist to a cell or animal in need thereof.

The methods of the invention can be used to treat any condition wherein it is desirable to modulate IgA receptor (such as pIgR or FcαR) activity in order to prevent calcium signalling and thereby inhibit a variety of calcium dependent effects in airway smooth muscle cells. Such conditions include, but are not limited to asthma, chronic bronchitis, acute bronchitis, bronchial hyperreactivity, chronic obstructive pulmonary disease, emphysema, interstitial lung disease, bronchiectasis or airway remodelling.

Accordingly, the present invention provides a method of treating a condition wherein it is desirable to inhibit a calcium dependent effect in an airway smooth muscle cell comprising administering an effective amount of an IgA receptor antagonist to an animal in need thereof.

As used herein, and as well understood in the art, "treating" is an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treating" can also mean prolonging survival as compared to expected survival if not receiving treatment.

In a preferred embodiment, the method is useful in treating asthma. It is possible that IgA-induced calcium signalling may directly influence smooth muscle contraction and thereby contribute to bronchial hyperreactivity in

asthma. It is also possible that IgA causes activation of transcription factors (e.g. NFκB) important for inflammatory reactions and subsequent production of cytokines, chemokines, adhesion molecules and growth factors. As a result, inhibition of IgA-induced calcium signalling may greatly improve the degree of inflammation in asthma as well as the airway remodelling described in chronic asthmatics. Furthermore, it is possible that this IgA-related phenomenon may account, at least in part, for the wide variety of clinical presentations and therapeutic responsiveness in patients with asthma.

Accordingly, the present invention provides a method of treating a patient with asthma comprising administering an effective amount of an IgA receptor antagonist to a patient in need thereof.

II. <u>Agents That Modulate pIgR or FcαR</u>

The finding by the present inventors that pIgR or FcαR are on airway smooth muscle cells allows the discovery and development of agents that modulate pIgR or FcαR for use in modulating diseases mediated through the pIgR or FcαR and/or through intracellular calcium signalling on airway smooth muscle cells.

The present invention includes the use of any and all agents that modulate pIgR or FcαR on airway smooth muscle cells in the methods of the invention. The agent can be any type of substance, including, but not limited to, nucleic acids (including antisense oligonucleotides, proteins (including antibodies), peptides, peptide mimetics, carbohydrates, organic compounds, inorganic compounds, small molecules, drugs, pIgR or FcαR ligands, soluble forms of pIgR or FcαR, pIgR or FcαR agonists, pIgR or FcαR antagonists, agents that inhibit pIgR or FcαR agonists, polymeric IgA (pIgA), dimeric IgA (dIgA) and polymeric IgM (pIgM) and fragments of these IgA or IgM molecules. Examples of some of the agents that modulate pIgR or FcαR are provided below.

(i) Antibodies

In one embodiment, the agent that can modulate pIgR is an antibody that binds to pIgR. Within the context of the present invention, antibodies are

understood to include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, Fab', $F(ab')_2$, scFv and Fv fragments) and recombinantly produced binding partners. Antibodies to pIgR may act as pIgR agonists or pIgR antagonists. For example, whole antibodies may act as pIgR agonists by stimulating the receptor while antibody fragments may act as pIgR antagonists by blocking the ability of pIgR ligands (such as pIgA) to bind pIgR.

In one embodiment, the antibody is an antibody fragment that acts as a pIgR antagonist. In a specific embodiment, the antibody fragment is a single chain Fv antibody. Single chain-Fv fragments can be isolated from a phage display library. The preparation of scFv antibodies to pIgR is described in Example 2.

In one embodiment, the agent that can modulate FcαR is an antibody that binds to FcαR. Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, Fab', $F(ab')_2$, scFv and Fv fragments) and recombinantly produced binding partners. Antibodies to FcαR may act as FcαR agonists or FcαR antagonists. For example, whole antibodies may act as FcαR agonists by stimulating the receptor while antibody fragments may act as FcαR antagonists by blocking the ability of FcαR ligands (such as mIgA or pIgA) to bind FcαR.

In one embodiment, the antibody is an antibody fragment that acts as a FcαR antagonist. In a specific embodiment, the antibody fragment is a single chain Fv antibody. Single chain-Fv fragments can be isolated from a phage display library. The preparation of scFv antibodies to FcαR is described in Example 2.

In another embodiment, the antibody is a pIgR agonist. Examples of antibodies that are pIgR agonists include pIgA and pIgM. Antibodies to pIgR may be prepared using techniques known in the art such as those described by Kohler and Milstein, Nature 256, 495 (1975) and in U.S. Patent Nos. RE 32,011; 4,902,614; 4,543,439; and 4,411,993, which are incorporated herein by reference. (See also Monoclonal Antibodies, Hybridomas: A New

Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference).

In another embodiment, the antibody is a FcαR agonist. Examples of antibodies that are FcαR agonists include mIgA and pIgA. Antibodies FcαR may be prepared using techniques known in the art such as those described by Kohler and Milstein, Nature 256, 495 (1975) and in U.S. Patent Nos. RE 32,011; 4,902,614; 4,543,439; and 4,411,993, which are incorporated herein by reference. (See also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference).

(ii) Antisense oligonucleotides

In another embodiment, the agent that can modulate pIgR or FcαR is an antisense oligonucleotide that acts as a pIgR or FcαR antagonist, respectively, by inhibiting the expression of the pIgR or FcαR gene. The term "antisense oligonucleotide" as used herein means a nucleotide sequence that is complimentary to its target, e.g. the pIgR or FcαR gene. The sequence of the pIgR and FcαR genes are known in the art for many species, for example, see Piskurich et al., *J. Immunol.* 154:1735-1747, 1995, and Maliszewski et al, *J. Exp. Med.* 172:1665-1672, 1990.

The term "oligonucleotide" as used herein refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric

oligonucleotides which contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into cells), or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

The antisense oligonucleotides of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

Other antisense oligonucleotides of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the antisense oligonucleotides may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates. In an embodiment of the invention there are phosphorothioate bonds links between the four to six 3'-terminus bases. In another embodiment phosphorothioate bonds link all the nucleotides.

The antisense oligonucleotides of the invention may also comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. An example of an oligonucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyamide backbone which is similar to that found in peptides (P.E. Nielsen, et al Science 1991, 254, 1497). PNA analogues have been shown to be resistant to degradation by enzymes and

to have extended lives in vivo and in vitro. PNAs also bind stronger to a complimentary DNA sequence due to the lack of charge repulsion between the PNA strand and the DNA strand. Other oligonucleotides may contain nucleotides containing polymer backbones, cyclic backbones, or acyclic backbones. For example, the nucleotides may have morpholino backbone structures (U.S. Patent No. 5,034,506). Oligonucleotides may also contain groups such as reporter groups, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an antisense oligonucleotide. Antisense oligonucleotides may also have sugar mimetics.

The antisense nucleic acid molecules may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The antisense nucleic acid molecules of the invention or a fragment thereof, may be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed with mRNA or the native gene e.g. phosphorothioate derivatives and acridine substituted nucleotides. The antisense sequences may be produced biologically using an expression vector introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense sequences are produced under the control of a high efficiency regulatory region, the activity of which may be determined by the cell type into which the vector is introduced.

(iii) Peptide Mimetics

The present invention also includes peptide mimetics of the pIgR or FcαR proteins. Such peptides may include competitive inhibitors, enhancers, peptide mimetics, and the like. All of these peptides as well as molecules substantially homologous, complementary or otherwise functionally or structurally equivalent to these peptides may be used for purposes of the present invention.

"Peptide mimetics" are structures which serve as substitutes for peptides in interactions between molecules (See Morgan et al (1989), Ann.

Reports Med. Chem. 24:243-252 for a review). Peptide mimetics include synthetic structures which may or may not contain amino acids and/or peptide bonds but retain the structural and functional features of a pIgR peptide, or enhancer or inhibitor of the pIgR peptide. Peptide mimetics also include peptoids, oligopeptoids (Simon et al (1972) Proc. Natl. Acad, Sci USA 89:9367); and peptide libraries containing peptides of a designed length representing all possible sequences of amino acids corresponding to a peptide of the invention.

Peptide mimetics may be designed based on information obtained by systematic replacement of L-amino acids by D-amino acids, replacement of side chains with groups having different electronic properties, and by systematic replacement of peptide bonds with amide bond replacements. Local conformational constraints can also be introduced to determine conformational requirements for activity of a candidate peptide mimetic. The mimetics may include isosteric amide bonds, or D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule. Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states. The mimetics can also include mimics of inhibitor peptide secondary structures. These structures can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins. Peptoids may also be used which are oligomers of N-substituted amino acids and can be used as motifs for the generation of chemically diverse libraries of novel molecules.

(iv) Other substances

In addition to antibodies and antisense oligonucleotides, other substances that can modulate pIgR or FcαR can also be identified and used in the methods of the invention. In one embodiment, the pIgR or FcαR modulator is a protein or peptide that can bind to pIgR or FcαR. The pIgR- or FcαR-binding peptides may be isolated by assaying a sample for peptides that bind to pIgR or FcαR. Any assay system or testing method that detects protein-protein interactions may be used including co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic

columns may be used. Biological samples and commercially available libraries may be tested for pIgR- or FcαR-binding peptides. For example, labelled pIgR or FcαR may be used to probe phage display libraries. In addition, antibodies that bind pIgR or FcαR may be used to isolate other peptides with pIgR or FcαR binding affinity. For example, labelled antibodies may be used to probe phage display libraries or biological samples. Additionally, a DNA sequence encoding a pIgR protein may be used to probe biological samples or libraries for nucleic acids that encode pIgR- or FcαR-binding proteins.

Substances which can bind pIgR or FcαR may be identified by reacting pIgR or FcαR, respectively, with a substance which potentially binds to pIgR or FcαR, then detecting if complexes between the respective receptor and the substance have formed. Substances that bind pIgR or FcαR in this assay can be further assessed to determine if they are useful in modulating or inhibiting pIgR or FcαR and useful in the therapeutic methods of the invention.

Accordingly, the present invention also includes a method of identifying substances which can bind to pIgR or FcαR comprising the steps of:

(a) reacting pIgR or FcαR and a test substance, under conditions which allow for formation of a complex between the pIgR or FcαR and the test substance, and

(b) assaying for complexes of pIgR or FcαR and the test substance, for free substance or for non complexed pIgR or FcαR, wherein the presence of complexes indicates that the test substance is capable of binding pIgR or FcαR.

Conditions which permit the formation of substance and IgA receptor complexes may be selected having regard to factors such as the nature and amounts of the substance and the protein.

The substance-IgA receptor complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or

combinations thereof. To facilitate the assay of the components, antibody against pIgR or FcαR or the substance, or labelled pIgR or FcαR, or a labelled substance may be utilized. The antibodies, pIgR or FcαR, or substances may be labelled with a detectable substance.

The pIgR or FcαR or the test substance used in the method of the invention may be insolubilized. For example, the pIgR or FcαR or substance may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, silica, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc.

The insolubilized pIgR or FcαR or substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

The pIgR or FcαR or test substance may also be expressed on the surface of a mesenchymal cell in the above assay.

The pIgR or FcαR gene or protein may be used as a target for identifying lead compounds for drug developments. The invention therefore includes an assay system for determining the effect of a test compound or candidate drug on the activity of the pIgR or FcαR gene or protein.

Accordingly, the present invention provides a method for identifying a compound that modulates pIgR or FcαR activity comprising:

(a) incubating a test compound with pIgR or FcαR protein or a nucleic acid encoding the pIgR or FcαR protein; and

(b) determining the effect of the test compound on the pIgR or FcαR protein activity or pIgR or FcαR gene expression and comparing with a control (i.e. in the absence of a test compound) wherein a change in the pIgR or FcαR protein activity or pIgR or FcαR gene expression as compared to the control indicates that the test compound is a potential modulator of the pIgR or FcαR gene or protein.

In one embodiment, pIgR or FcαR activity may be assessed by measuring intracellular calcium levels as previously described.

III. Compositions

The present invention also includes pharmaceutical compositions containing the agents that can modulate or inhibit an IgA receptor (such as pIgR or FcαR) for use in the methods of the invention. Accordingly, the present invention provides a pharmaceutical composition for modulating calcium signalling in an airway smooth muscle cell comprising an effective amount of an agent that can modulate an IgA receptor in admixture with a suitable diluent or carrier. The present invention also includes a pharmaceutical composition for preventing or inhibiting calcium signalling in an airway smooth muscle cell comprising an effective amount of an IgA receptor antagonist in admixture with a suitable diluent or carrier. The present invention further provides a pharmaceutical composition for preventing or treating asthma comprising an effective amount of an IgA receptor antagonist in admixture with a suitable diluent or carrier. In a preferred embodiment, the IgA receptor antagonist is a pIgR or FcαR antagonist.

Such pharmaceutical compositions can be for intralesional, intravenous, topical, rectal, parenteral, local, inhalant or subcutaneous, intradermal, intramuscular, intrathecal, transperitoneal, oral, and intracerebral use. The composition can be in liquid, solid or semisolid form, for example pills, tablets, creams, gelatin capsules, capsules, suppositories, soft gelatin capsules, gels, membranes, tubelets, solutions or suspensions.

The pharmaceutical compositions of the invention can be intended for administration to humans or animals. Dosages to be administered depend on individual needs, on the desired effect and on the chosen route of administration.

The pharmaceutical compositions can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in

Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

On this basis, the pharmaceutical compositions include, albeit not exclusively, the active compound or substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids. The pharmaceutical compositions may additionally contain other agents such as other agents that can modulate or inhibit calcium signalling or that are used in treating asthma.

IV. Targeted Delivery

The finding by the present invention that pIgR and FcαR are on airway smooth muscle cells allows the development of methods to target the delivery of substances directly to airway smooth muscle cells. Accordingly, the present invention provides a method of delivering a substance to an airway smooth muscle cell comprising administering an effective amount of a conjugate comprising the substance coupled to an IgA receptor ligand to an animal or cell in need thereof.

The substance can be any substance that one wishes to deliver, including therapeutics and diagnostics, to an airway smooth muscle cell. In a specific embodiment, the substance is useful in treating asthma. In another embodiment, the substance is useful in diagnosing IgA receptor-mediated asthma.

The ligand can be any molecule that can bind an IgA receptor (such as pIgR or FcαR) including pIgA or pIgM as well as the ligands described in Section II.

The substance may be coupled to the IgA receptor ligand either directly or indirectly. In direct coupling, the substance and ligand are physically linked such as by chemical or recombinant covalent binding or by physical forces such as van der Waals or hydrophobic or hydrophilic interactions. In indirect coupling, the substance and ligand are joined through another molecule or linker. As one example, the substance and ligand may be joined through a recombinant bispecific antibody that binds both the substance and linker.

Conjugates of the substance and the IgA receptor ligand may be prepared using techniques known in the art. There are numerous approaches for the conjugation or chemical crosslinking of proteins and one skilled in the art can determine which method is appropriate for the substance to be conjugated. The method employed must be capable of joining the substance with the IgA receptor ligand without interfering with the ability of the ligand to bind to the IgA receptor and without significantly altering the activity of the substance. If the substance and ligand are both proteins, there are several hundred crosslinkers available in order to conjugate the substance with the ligand. (See for example "Chemistry of Protein Conjugation and Crosslinking". 1991, Shans Wong, CRC Press, Ann Arbor). The crosslinker is generally chosen based on the reactive functional groups available or inserted on the substance. In addition, if there are no reactive groups a photoactivatible crosslinker can be used. In certain instances, it may be desirable to include a spacer between the substance and the ligand. In one example, the ligand and substance may be conjugated by the introduction of a sulfhydryl group on the ligand and the introduction of a cross-linker containing a reactive thiol group on to the substance through carboxyl groups (Wawizynczak, E.J. and Thorpe, P.E. in Immunoconjugates: Antibody Conjugates in Radioimaging and Therapy of Cancer, C.W. Vogel (Ed.) Oxford University Press, 1987, pp. 28-55.; and Blair, A.H. and T.I. Ghose, J. Immunol. Methods 59:129 ,1983).

In another embodiment, the protein ligand and substance may be prepared as a fusion protein. Fusion proteins may be prepared using techniques known in the art. In such a case, a DNA molecule encoding the IgA receptor ligand or antagonist is linked to a DNA molecule encoding the substance. The chimeric DNA construct, along with suitable regulatory elements can be cloned into an expression vector and expressed in a suitable host.

The conjugates of the invention may be tested for their ability to enter mesenchymal cells and provide the desired pharmacological effect using *in vitro* and *in vivo* models.

V. Diagnostic Assays

The finding by the present inventors that airway smooth muscle cells have IgA receptors (such as pIgR and FcαR) allows the development of diagnostic assays to detect IgA mediated diseases. Such diagnostic assays can facilitate the development of tailored therapies for such diseases. In one example, patients can undergo bronchial challenge testing to determine the presence of IgA-mediated bronchial hyperreactivity. Methacholine challenge and histamine challenge testing are examples of currently used tests to evaluate respiratory symptoms of cough, wheeze and shortness of breath, and to detect nonspecific bronchial hyperresponsiveness as demonstrated by exaggerated bronchoconstriction to inhaled methacholine or histamine. Pre- and post-challenge pulmonary function test measurements of forced expiratory volume at 1 second (FEV1) and forced vital capacity (FVC) could be done every 5-10 minutes following bronchial challenge with an agonist to IgA receptors that would results in increased intracellular calcium concentrations in ASM and consequent bronchoconstriction. The IgA receptor agonist can be any substance that will increase airway smooth muscle calcium concentrations and contraction which would manifest clinically as bronchoconstriction. This bronchoconstriction can be detected subjectively by hearing wheezing on chest auscultation, or objectively by measuring a reduced FEV1 by standard spirometry. Fo example, bronchoconstriction may be detected by a drop in FEV1. IgA-mediated bronchial hyperreactivity could be graded according to the concentration of test substance that results in a 20% fall in baseline FEV1. Accordingly, the present invention provides a method of detecting IgA mediated bronchial hyperreactivity comprising:

(a) administering an IgA receptor agonist to a patient; and

(b) detecting bronchoconstriction in the patient wherein an increase in bronchoconstriction as compared to a control indicates that the patient has IgA-mediated hyperreactivity.

Another example of a diagnostic test would be to perform a nonspecific bronchial challenge test with either methacholine or histamine on one day, and then repeat this challenge on another day after pretreating patients with

an IgA receptor agonist. IgA-mediated bronchial hyperreactivity would be detected by a significant increase sensitivity to the non-specific bronchoconstrictor (i.e. a lower dose of methacholine or histamine induces the 20% fall in FEV1 from baseline measurement). Another example would be to perform a bronchial challenge test with both a nonspecific bronchoconstrictor and an IgA receptor agonist.

Accordingly, the present invention provides a method of detecting IgA-mediated bronchial hyperreactivity comprising:

(a) administering an IgA-receptor agonist to a patient and detecting bronchoconstriction; and

(b) administering an IgA receptor agonist followed by a non-specific bronchoconstricting agent to the patient at a lower dose than when the nonspecific agent is administered alone and detecting bronchoconstriction wherein bronchoconstriction in step (a) and/or bronchoconstriction induced at a lower dose of the nonspecific agent administered without the IgA receptor agonist in step (b) would indicate that the patient has IgA-mediated bronchial hyperreactivity.

In a bronchial challenge test, a patient inhales increasing amounts of the agent. The patient's FEV1 and FVC are measured by spirometry 30-90 seconds after inhaling the agent. If there is no significant (= 20% change from baseline), the next dose is administered 5 minutes later, but no sooner than 5 minutes. The challenge is stopped when the patient develops a 20% drop in baseline FEV1 (- which indicates bronchoconstriction - or FVC) or a specific maximum dose has been given (=8 mg/ml in the case of methacholine). (Higher doses of methacholine will cause bronchoconstriction in nearly everyone. People who react to doses less than 8 mg/ml are categorized as having severe, moderate, mild or borderline hyperreactivity to the agent depending on the dose of the agent that causes the 20% drop in FEV1. These challenge tests are used to diagnose bronchial hyperreactivity and monitor therapy. Note that narrowing might occur in very distal airways without narrowing in more proximal airways and that this very distal narrowing might not result in changes in FEV1.

In an alternative approach, samples from patients can be obtained and tested for the presence of IgA receptors, such as pIgR or FcαR, on airway smooth muscle cells. The sample can be any sample that contains airway smooth muscle cells including transbronchial biopsies and lung biopsies. Alternatively, soluble or secreted forms of the airway smooth muscle IgA receptor may be detected in fluid samples,including sputum (spontaneously produced), induced sputum, bronchoalveolar lavage fluid, serum or nasal washings. Patients whose samples show evidence ofan airway smooth muscle IgA receptor may be treated with IgA receptor antagonists as described above. This approach to detect IgA receptors could be combined with a functional test as described above for bronchial challenges.

Accordingly, the present invention provides a method of detecting a condition associated with the activation of an IgA receptor on an airway smooth muscle cell comprising assaying a sample for (a) a nucleic acid molecule encoding an IgA receptor or a fragment thereof or (b) an IgA receptor or a fragment thereof. One possibility is that a restriction fragment length polymorphism (RFLP) in the IgA receptor is associated with an IgA receptor mediated disorder or condition. The IgA receptor is preferably pIgR or FcαR. In one embodiment, the condition associated with the activation of an IgA receptor on an airway smooth muscle cell is asthma, chronic bronchitis, acute bronchitis, bronchial hyperreactivity, chronic obstructive pulmonary disease, emphysema, interstitial lung disease, bronchiectasis or airway remodelling.

(i) Detecting Nucleic acid molecules encoding IgA receptors

Nucleotide probes can be prepared and used in the detection of nucleotide sequences encoding an IgA receptor or fragments thereof in samples, preferably pIgR or FcαR. The probes can be useful in detecting the presence of a condition associated with the activation of an IgA receptor on an airway smooth muscle cell or monitoring the progress of such a condition. Such conditions include asthma, chronic bronchitis, acute bronchitis, bronchial hyperreactivity, chronic obstructive pulmonary disease, emphysema, interstitial lung disease, bronchiectasis or airway remodelling. Accordingly,

the present invention provides a method for detecting a nucleic acid molecule encoding an IgA receptor comprising contacting the sample with a nucleotide probe capable of hybridizing with the nucleic acid molecule to form a hybridization product, under conditions which permit the formation of the hybridization product, and assaying for the hybridization product.

The nucleotide probe may be labelled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as 32P, 3H, 14C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and chemiluminescence. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleic acid to be detected and the amount of nucleic acid available for hybridization. Labelled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.).

Nucleic acid molecules encoding an IgA receptor can be selectively amplified in a sample using the polymerase chain reaction (PCR) methods and cDNA or genomic DNA. A nucleic acid can be amplified from cDNA or genomic DNA using oligonucleotide primers and standard PCR amplification techniques. The amplified nucleic acid can be cloned into an appropriate vector and characterized by DNA sequence analysis. cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

(ii) Detecting IgA receptors

The presence of IgA receptors may be detected in a sample using IgA receptor ligands that bind to the IgA receptor. IgA receptor ligands are described above and include antibodies or other substances that can bind an IgA receptor. Accordingly, the present invention provides a method for detecting an IgA receptor comprising contacting the sample with a ligand that binds to an IgA receptor which is capable of being detected after it becomes bound to the IgA receptor in the sample.

Ligands to an IgA receptor, such as antibodies specifically reactive with an IgA receptor, or derivatives thereof, such as enzyme conjugates or labeled derivatives, may be used to detect an IgA receptor in various biological materials. For example they may be used in any known immunoassays which rely on the binding interaction between an IgA receptor, and an antibody thereof. Examples of such assays are radioimmunoassays, enzyme immunoassays (e.g. ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination and histochemical tests. Thus, the antibodies may be used to detect and quantify an IgA receptor in a sample in order to determine its role in particular cellular events or pathological states, and to diagnose and treat such pathological states, such as asthma.

Cytochemical techniques known in the art for localizing antigens using light and electron microscopy may be used to detect an IgA receptor. Generally, an antibody of the invention may be labelled with a detectable substance and an IgA receptor may be localised in tissue based upon the presence of the detectable substance. Examples of detectable substances include various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include radioactive iodine I-125, I-131 or 3-H. Antibodies

may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against an IgA receptor. By way of example, if the antibody having specificity against an IgA receptor is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labelled with a detectable substance as described herein.

Where a radioactive label is used as a detectable substance, an IgA receptor may be localized by autoradiography. The results of autoradiography may be quantitated by determining the density of particles in the autoradiographs by various optical methods, or by counting the grains.

Bioassays would constitute an additional diagnostic approach. For example, fluid samples obtained from patients (as described above) that might contain IgA receptor agonists could be incubated with Fura-2 loaded cells that express IgA receptors, including primary or transformed smooth muscle cells. These cells could be grown on 96 well plates and monitored in a kinetic fluorescence plate reader with a heated stage for changes in intracellular calcium concentrations in response to patient samples.

Diagnostic bioimaging could be developed using inhaled or injected radio-labeled or chemically-conjugated ligands to IgA receptors that do not stimulate intracellular calcium changes. Binding to ASM could be detected using standard radiography or CT scanning with iodine-labeled ligands to IgA receptors, or nuclear scanning to detect Technetium-labeled ligands, or nuclear magnetic resonance imaging of gadolinium-labeled ligands.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1

METHODS

Cell culture:

Primary human airway smooth muscle (ASM) cells from four different subjects were purchased from Clonetics (San Diego, CA, USA) and grown in smooth muscle cell basal medium (SmBM) (Clonetics) supplemented with the smooth muscle cell SingleQuots (Clonitics), which containing 0.5 ng/ml human recombinant Epidermal Growth Factor (hEGF), 5µg/ml insulin, 1µg/ml human recombinant Fibroblast Growth Factor (hFGF), 50µg/ml Gentamicin and 50 ng/ml Amphotericin-B, and 5% fetal calf serum. At 75% confluence, the cells were serum-deprived to induce differentiation (Halayko et al, 1999) and grown in serum-free basal media (Clonetics). Cells were studied between days 8 and 14 after serum starvation. ASM phenotype was confirmed by positive staining for markers of smooth muscle cell differentiation including myosin kinase light chain, and negative staining for factor VIII. ASM cells were used within the first 9 passages and perpetuated by trypsinizing cells for propagating at a 1:4 dilution.

Madin Darby canine kidney (MDCK) cells transfected with the cDNA for human pIgR were used as positive control cells (MDCK-HpIgR, from Dr. Charlotte Kaetzel) for detection of pIgR, and the myelomonocytic cell line, U937 (ATCC), was used for its high level of endogenous expression of Fc-alpha receptor.

Immunofluorescence studies:

ASM are grown on collagen-coated coverslips in serum-containing growth medium to 70% confluence and then replaced with serum-free medium for 8 to 14 days. After 16 hours treatment with pIgA, mIgA, rabbit anti-human secretory component (SC; Dako) or media alone, the coverslips were washed twice with ice cold PBS and fixed with 4% paraformaldehyde in PBS for 20 min on ice. After washed three times in PBS, the coverslips were blocked with 5% horse serum in PBS and 0.2% triton X-100 for 1h at 37°C. Goat anti-alpha chain or rabbit anti-human SC at 1:100 dilution in blocking buffer were incubated with the coverslips for 1 h at 37°C. After washed three times in PBS and 0.05% triton X-100, the coverslips were incubated with secondary antibody FITC-labeled donkey anti-goat or donkey anti-rabbit at 1:200 dilution in blocking buffer for 45 min at 37°C. The coverslips then were

mounted onto slides with one drop of mounting medium (Vector laboratories, Burlingame, CA, USA) and Fluorescence images were observed and captured with an Olympus fluorescence microscope with a digital camera.

Protein and RNA expression:

Western blotting. ASM cells were lysed in 0.5% SDS lysis buffer with protease inhibitor (100mM NaCl, 50mM Tris [pH 8.1), 5mM EDTA [pH8.0], 0.02 % NaN_3, 0.5 % SDS, 1mM PMSF, 10µg/ml aprotinin, 1mM Na_3VO_4) and run on a 8% SDS-PAGE gel. Lysates of MDCK-HpIgR were used as a positive control. After transferring to nitrocellulose membrane (Bio-Rad) for 1.5h at 100 voltage in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol), Western blotting was performed using either a rabbit anti-human secretory component antibody (Dako) or a goat anti-human secretory component antibody (Sigma). The anti-SC antibody was detected with an HRP-labeled secondary goat antibody to rabbit IgG (Sigma) or donkey antibody to goat (Jackson Immunochemicals). Antibody binding was detected on film (Amersham Pharmacia Biotech) using enhanced chemiluminescence (ECL) (Amersham-Pharmacia Biotech).

RT-PCR and DNA sequencing. For detecting mRNA expression of pIgR or Fc-alpha receptor, total RNA was extracted from ASM, MDCK-HpIgR or U937 cells with TRIzol reagent (Life Technologies). Total RNA extracted from MDCK-HpIgR cells were used as a positive control for pIgR, while the U937 cells were used as the positive control for Fc-alpha receptor. Primers were designed to represent the cytoplasmic domain of pIgR and the IgA binding domain of the Fc-alpha receptor (Table 1 shows the sequence of the sense and antisense primers). RT-PCR was performed using oligo dT, mMLV reverse transcriptase and Taq DNA polymerase (Life Technologies). The RT-PCR products were run on a 1.2 % agarose gel. Bands from the ASM lanes were cut out and purified with QIAquick Gel Extraction kit (Qiagen) for DNA sequencing. DNA sequences were compared to those in the Genebank of NCBI human genome database for identification.

Calcium studies:

(1) Calcium measurements. ASM were grown in serum free media for 8 days on 96-well fluorescence plates (Costar). After loading cells with Fura 2-AM (Molecular Probe) for 3h at 37°C, the ASM cells were treated with either media alone, mIgA (12μg/ml) or pIgA (12μg/ml). Fluorescence was measured using a multiwell fluorescence plate reader (Molecular Dynamics) at excitation wavelengths of 340λ and 380λ and emission wavelengths at 500λ. Data was collected and calculated calcium concentration using the Grynkiewicz formula: $[Ca^{2+}]_i$ (in nmol/L) = Kd $[(R-R_{min})/(R_{max}-R)]x\beta$ factor. R is the ratio of fluorescence at 340 and 380 nm, R_{max} and R_{min} are the ratios at 340 and 380 nm in the presence of saturating Ca^{2+} and zero Ca^{2+}, β factor is the ratio at 380 nm in zero and saturating Ca^{2+}, and Kd , the dissociation constant , is 224 nmol/L.

(2) Calcium imaging. Fluorescence spectrophotometric and imaging techniques were used to study calcium signaling in Fura-2 loaded primary human ASM grown to 70% confluence on collagen-coated coverslips and serum-starved for 10-14 days. Cells were loaded with Fura-2 (Molecular Probes) for 1h at 37C. Cells were monitored for X min to ensure imaging stability, prior to being perfused with either (1) HEPES buffered KREBS solution containing 140mM NaCl, 4.9 mM KCl, 1.4mM KH_2PO_4, 1.2mM $MgCl_2$, 11mM Glucose, 25 mM HEPES, 2mM $CaCl_2$ at pH 7.4 and 37C; (2) pIgA (purchased from Dr. Vaerman) 0.12 - 12 μg/ml diluted in the KREBS buffer; or (3) mIgA (Pierce) 12 μg/ml in the KREBS buffer. Each cell was circled for automatic data processing of intracellular free calcium concentration for each cell and images were stored. Data was analyzed by Excel.

RESULTS

Primary ASM cell cultures express IgA receptors by immunofluorescence.

Human bronchial ASM cells from 4 different, non-asthmatic donors were obtained from Biowhitaker/Clonetics. All cells were used within 9 passages, and characterized by positive immunostaining with smooth muscle-specific actin and myosin, but not with factor VIII which recognizes endothelial

cells. Primary cell cultures of serum-starved human bronchial ASM grown on coverslips were fixed and stained for pIgR with antibody to human SC, the extracellular portion of pIgR. Figure 1A confirms green staining for pIgR in one representative ASM sample from the 4 different subjects. In another experiment, serum-starved ASM were incubated overnight with pIgA or mIgA, washed extensively, fixed with paraformaldehyde and then immuno-stained with antibodies to the alpha chain of IgA. Only ASM incubated with pIgA immunostained for the alpha chain (Figure 1B, left panel), in contrast to ASM incubated with mIgA which does not bind pIgR (Figure 1B, right panel). Live uptake experiments were also performed on serum-starved ASM pre-incubated with media alone (negative control), rabbit antibody to SC (ligand for pIgR), or an irrelevant rabbit IgG (control antibody). The cells were washed, fixed and stained with a FITC-conjugated secondary antibody to rabbit IgG. Figure 1C confirms live-uptake of the rabbit antibody to SC (right panel) in contrast to the irrelevant IgG (middle panel) or serum alone.

These results show that serum-starved ASM express the pIgR protein and that it is capable of selectively binding its ligands (pIgA or rabbit antibody to SC) and not irrelevant ligands (mIgA or irrelevant rabbit IgG).

Figure 2 shows that in contrast to serum-starved ASM, non-starving ASM do immunostain for the FcαR (top panel). In addition, this FcαR expression increases after pre-incubation of live cells with either pIgA (middle panel) or mIgA (lower panel), both of which can bind the FcαR.

Thus Figures 1 and 2 confirm that ASM cells express both the pIgR and the FcαR protein. Furthermore, ligands to FcαR upregulate surface expression of this receptor on non-starving ASM.

Primary cultures of ASM have IgA receptors detected by western blotting.

Figure 3 shows a western blot that confirms the presence of pIgR in both serum-starved and non-starving ASM. MDCK-HpIgR epithelial cells were used as a positive control for pIgR protein expression. The results of these western blots confirmed the presence of a band consistent with pIgR (Figure 3, upper panel). It is of interest that the pIgR band from the 12-day serum-

starved ASM appeared as a singlet, in contrast to the pIgR band from transfected MDCK cells, which appears as a doublet due to phosphorylation of the receptor. Non-starving ASM expressed the doublet pIgR band, as do the 6-day starving cells (Figure 3, upper panel). Stripping this blot and re-probing with 4G10 (UBI) monoclonal antibody to phospho-tyrosine shows that the second band of pIgR is phosphorylated in non-starving ASM and in transfected MDCK cells, as commonly found with many receptors (Figure 3, lower panel).

Figure 3 adds further evidence that ASM express pIgR protein and that it appears as a doublet due to phosphorylation when the cells are grown with serum.

Primary cultures of ASM have IgA receptors detected by RT-PCR

Figure 4 shows that ASM have mRNA encoding the cytoplasmic domain of pIgR by RT-PCR. MDCK-HpIgR cells were used as a positive control for pIgR mRNA message. DNA sequencing of this band (339 bp) revealed partial sequence homology to the epithelial pIgR. These results confirm that ASM express mRNA for pIgR.

Figure 5 shows that serum-starved ASM do not have mRNA encoding the IgA-binding domain of FcαR by RT-PCR (right lane). In contrast, RT-PCR with non-starving cells showed that ASM do express FcαR (Figure 5, middle lane). U937 cells were used as a positive control for FcαR mRNA message. DNA sequencing of the RT-PCR product from non-starving ASM (241 bp) confirmed it is the FcαR.

These results show that ASM express mRNA for both pIgR and FcαR. Furthermore, serum-deprivation down-regulates mRNA expression for FcαR in ASM.

Polymeric IgA increases intracellular free calcium concentrations in ASM.

Incubation with pIgA significantly increased intracellular free calcium concentrations in 8 day serum-starved ASM in contrast to mIgA or media alone (Table 2). The effect of pIgA was seen after 1h, peaked at 1h 15 min and was sustained for 1.5 h, which was the duration of the experiment.

Fluorescence spectrophotometric and imaging techniques confirmed these results. Incubation of serum-starved ASM with pIgA significantly increased intracellular calcium concentrations (Figure 6A), in contrast to incubation with buffer alone which shows no change in calcium (Figure 6B). In Figure 6, each line represents a different cell monitored for the duration of the experiment on the same day. Each graph represents one of 5 studies per condition. Of greatest interest, the pIgA-induced rise in intracellular free calcium concentrations occurred consistently after about 1.5h and manifested a sustained response with an oscillating pattern for the duration of the experiment.

Similar experiments were done after first testing the serum-starved ASM with histamine, a known stimulus for ASM contraction and bronchoconstriction. After the histamine was washed off, a low concentration of pIgA (0.12 µg/ml) was added. After 1h, intracellular calcium concentrations increased and rose 4-6 fold with repeat histamine stimulation after washing off pIgA (Figure 7A). The time control experiment in Figure 7B shows that pre-exposure to histamine sensitizes the serum-starved ASM cells to a second histamine exposure 2h later with less than a 2-fold increase in intracellular calcium concentration. When a similar experiment was done with a high dose of mIgA (12 µg/ml), re-exposure to histamine increased calcium levels less than 2-fold after washing of the mIgA (Figure 7C), as occurred with buffer alone in Figure 7B. Finally, repeating this experiment with a high dose of pIgA (12 µg/ml) after initial exposure to histamine and carbachol (another stimulus for ASM contraction and bronchoconstriction), caused a dramatic rise in intracellular calcium concentrations beginning 50 minutes later and going off-scale at 66 minutes with over a 30-fold increase (Figure 7D).

These results show a unique response of ASM to pIgA that has never been described before. First, pIgA causes a consistent delayed rise in intracellular calcium concentrations. This delayed response provides a window of opportunity to potentially alter cell signaling events triggered by the rise in intracellular calcium concentrations. Second, pIgA causes a sustained rise in calcium concentrations that oscillate. The frequency of these

oscillations have been associated with increasing the activity of the pro-inflammatory transcription factor, NFκB (Hu et al, *J. Biol. Chem.* 274:33995-33998, 1999). Chronic airway inflammation characterizes asthma and has been associated with airway remodeling that leads to irreversible changes. Third, histamine appears to sensitize ASM to pIgA. This particular finding potentially has major implications for treatment of allergic asthmatics who develop upper airway infections. Fourth, the lack of response to mIgA and absence of FcαR in non-starving ASM indicate that pIgA mediates its effect via pIgR.

Finally, imaging of serum-starved ASM exposed to the highest concentration of pIgA (12 μg/ml) during the Fura-2 calcium experiments shows ASM contraction (Figure 8). The cell that is circled in Frame 1 contracts and disappears by Frame 11. Not all the cells respond at the exact same time or to the same degree. However, even the cell in the lower right hand corner also changes shape. These cells were grown on collagen coverslips and were noticeably subconfluent, and may account for the differences in time and intensity of response. Cell contraction has never been described before in response to pIgA. The fact that this happens in serum-starved cells, again, implies that this pIgA effect is mediated via pIgR.

Example 2

scFv selection methods and results:

A scFv phage library was reconstituted by pooling all first rounds of selection that the inventor had previously prepared. The scFv phage library that was originally used is described in: Sheets MD, Amersdorfer P, Finnern R, Sargent P, Lindquist E, Schier R, Hemingsen G, Wong C, Gerhart JC, Marks JD, Lindqvist E., Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. (*Proc Natl Acad Sci U S A.* 1998 May 26;95(11):6157-62.).

TG-1/pHen /phage$^{1st\ round}$ scFv. These selections were to the domain 6 of rat pIgR; and to cell selections for pIgR with MDCK cells transfected with rabbit pIgR and attempted in 12 different ways. These TG-1 from 13 tubes

were combined and grown for isolating phage. These phage were used as the "reconstituted" phage library of scFv.

A. For selections against the ASM FcαR:

1. Coat 3 immunotubes with mIgA (Biolynx; 6.5 λ/3 ml PBS) and block with 2% milk.

2. Preclear reconstituted phage library twice with 2 of the coated immunotubes.

3. Incubate SDS lysates from non-serum-starved ASM (purchased from Biowhittaker) with the 3rd coated tube (- to bind the putative ASM FcαR to the mIgA).

4. Incubate precleared phage with the 3rd tube.

5. Wash extensively (15-20) with PBS and elute the bound phage with 1% TEA (triethanolamine). Neutralize the high pH with 1M Tris pH 7.4.

6. Infect TG-1 E. coli with the phage, and grow.

7. Expand and rescue phage to repeat procedure 2 more times.

8. After round 3, randomly pick 96 colonies and screen these for scFv production (dot blot) and cell ELISAs using (a) U937 cells (myelomonocytic cell line that highly expresses the FcαR); and (b) ASM, both cell lines grown in a 96-well plate.

Results:

- 8 positives by ASM ELISA (OD450>0.2); 3 positives by U937 cell ELISA
  - BstN1 DNA digest of pcr products from the 3 clones showed unique patterns, suggesting isolation of 3 different scFv

B. For selections against the ASM pIgR:

1. Coat 3 immunotubes with pIgA (10 λ myeloma serum/3 ml PBS) and block with 2% milk PBS.

2. Preclear reconstituted phage library twice with 2 of the coated immunotubes.

3. Incubate SDS lysates from non-serum-starved ASM (purchased from Biowhittaker) with the 3rd coated tube (- to bind the putative ASM pIgR to the pIgA).

4. Incubate precleared phage with the 3rd tube.

5. Wash extensively (15-20) with PBS and elute the bound phage with 1% TEA. Neutralize with 1M Tris pH 7.4.

6. Infect TG-1 with the phage, and grow.

7. Expand and rescue phage to repeat procedure 2 more times.

8. After round 3, randomly pick 96 colonies and screen these for scFv production (dot blot) and cell ELISA using ASM and CALU-3 cells grown in a 96-well plate.

Results:

- 45 positives by ASM ELISA (includes 6 that were negative on CALU-3); 55 positives by CALU-3 ELISA.
  - Also screened by ELISA with human milk which contains secretory component, the extracellular part of pIgR, and found 26 positives (used OD450>0.4 with background reading of ~0.1); screened by ELISA with fetal calf serum-coated plate, and found 46 positives; rabbit anti-human SC antibody (Dako) used as positive control antibody
  - BstN1 DNA digest of pcr products from the all positive clones showed 12 unique patterns, suggesting isolation of 12 different scFv

C. For selections against J-chain:

Dr. Jiri Mestecky sent his PET32 plasmid containing the J-chain protein fused to thioredoxin and containing an IgA protease cleavage site and a 6His tag for purification. This plasmid was infected into BL21 E. coli which were induced to produce the J-chain-thioredoxin fusion protein. This was purified by IMAC on a nickel resin.

1. Coat 2 immunotubes with thioredoxin (Sigma; 10 µg/ml) and block with 2% milk/PBS.

2. Coat 1 immunotube with the purified J-chain fusion protein, then block with 2%milk/PBS.

3. Preclear reconstituted phage library twice with the 2 thioredoxin-coated immunotubes.

4. Incubate precleared phage with the 3rd tube coated with the J-chain fusion protein.

5. Wash extensively (15-20) with PBS and elute the bound phage with TEA (triethanolamine). Neutralize the high pH with Tris buffer.

6. Infect [TG-1] E. coli with the phage, and grow.

7. Expand and rescue phage to repeat procedure 2 more times.

8. After round 3, randomly pick 96 colonies and screen these for scFv production (dot blot) and protein ELISA using one 96-well plate coated with thioredoxin and one plate coated with the J-chain fusion protein. (- To ensure that the scFv selected bind to J-chain and not to thioredoxin.)

Results:

- 30 positives by J-chain ELISA; none bound the thioredoxin-coated plate. (Background OD450 was ~0.07; chose OD450>0.2 to be positive.)

- 14 of these were induced to produce scFv and all recognized J-chain by western blotting (mouse monoclonal anti-J-chain from InnoGenex was used as positive control); 9E10 (anti-myc mouse monoclonal antibody and anti-mouse HRP alone used as negative control). (Figure 9)

- BstN1 DNA digest of pcr products from all positive clones showed 5 unique patterns, suggesting isolation of 5 different scFv.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

TABLE 1

Protein	Primer sequences
pIgR: cytoplasmic domain	
sense	5' GAC CCC ACT CCC TGC TCT AAC 3'
antisense	5' AGA AGA GGG GAA GGA CGG GAG 3'
FcαR: IgA binding domain	
sense	5' CCT CAG TCT GGG GCT TTC TTT 3'
antisense	5' CTT GTT TGC GTC CAT GTG GTC 3'

TABLE 2 – Intracellular calcium concentration (nM) in starving ASM at 100 min after adding stimulus:

Expt.	control	mIgA	pIgA*#
1	353.50	374.00	1556.00
2	501.46	1961.61	2481.45
3	640.25	853.74	556.78
4	537.25	995.72	1465.87
5	445.43	588.41	1391.39
means	**495.58**	**954.70**	**1490.30**
S.E.	**47.56**	**273.12**	**305.12**

T-TEST

P=0.08, mIgA versus control

*P=0.02 (P<0.05), pIgA versus control

#P=0.046 (P<0.05), pIgA versus mIgA

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

Ackermann LW, Wollenweber LA, Denning GM. IL-4 and IFN-γ increase steady state levels of polymeric Ig receptor mRNA in human airway and intestinal epithelial cells. *J. Immunol.* 162:5112-5118, 1999.

Bell DY, Haseman JA, Spock, A, McLennan G, Hook GER. Plasma proteins of the bronchoalveolar surface of the lung; smokers and nonsmokers. *Am. Rev. Respir. Dis.* 124: 72-79, 1981.

Childers NK, Bruce MG, McGhee JR. Molecular mechanisms of IgA defense. *Ann. Rev. Microbiol.* 43: 503-536,1989

Halayko AJ, Salari H, Ma X, Stephens NL. Markers of airway smooth muscle cell phenotype. *Am. J. Physiol.* 270:L1040-51, 1996.

Halayko AJ, Camoretti-Mercado B, Forsythe SM, Vieira JE, Mitchell JA, Wylam ME, et al. Divergent differentiation paths in airway smooth muscle culture: induction of functionally contractile myocytes. *Am. J. Physiol.* 276:L197-206.

Holgate ST, Davies DE, Lackie PM, Wilson SJ, Puddicomb SM, Lordan JL. Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J. Allergy Clin. Immunol.* 105:193-204, 2000.

Hu Q, Deshpande S, Irani K, Ziegelstein R. [Ca2+]I oscillation frequency regulates agonist-stimulated NFkB transcriptional activity. *J. Biol. Chem.* 274:33995-33998, 1999.

Kerr MA, Woof JM. Fc-alpha Receptors. In: Mucosal Immunology. PL Ogra, J Mestecky, M Lamm, W Strober, J Bienenstock, JR McGhee, editors. San Diego; Academic Press, 1999.

Leung JC, Tsang AW, Chan DT, Lai KN. Absence of CD89, polymeric immunoglobulin receptor, and asialoglycoprotein receptor on human mesangial cells. *J. Am. Soc. Nephrol.* 11:241-9, 2000.

Loman S, Radl J, Jansen HM, Out TA, Lutter R. Vectorial transcytosis of dimeric IgA by the Calu-3 human lung epithelial cell line: upregulation by IFN-gamma. *Am. J. Physiol.* 272:L951-8,1997.

Louis R, Shute J, Biagi, S, Stanciu L, Marrelli F, Tenor H, Hidi R, Djukanovic R. Cell infiltration, ICAM-1 expression, and eosinophil chemotactic activity in asthmatic sputum. *Am. J. Respir. Crit. Care Med.* 155:466-72, 1997.

Luton F, Verges M, Vaerman JP, Sudol M, Mostov KE. The SRC family protein tyrosine kinase p62yes controls polymeric IgA transcytosis *in vivo*. *Mol. Cell* 4:627-32, 1999.

Maliszewski CR, March CH, Schoenborn MA, Gimpel S, Shen L. Expression cloning of a human Fc receptor for IgA. *J. Exp. Med.* 172:1665-1672, 1990.

Mestecky J, Moro I, Underdown BJ. Mucosal Immunoglobulins. In: Mucosal Immunology. PL Ogra, J Mestecky, M. Lamm, W Strober, J Bienenstock, JR McGhee, editors. San Diego: Academic Press, 1999.

Morton H, Van Egmond M, Van de Winkel J. Structure and function of human IgA Fc receptors (F∀R). *Crit. Rev. Immunol.* 16: 423-440, 1996.

Mostov KE, Altschuler Y, Chapin SJ, Enrich C, Low SH, Luton F, Richman-Eisenstat J, Singer K, Tang K, Weimbs T. Regulation of protein traffic in

polarized epithelial cells: the pIgR model. Cold Spring Harbor Symposia on Quantitative Biology. Protein Kinesis: *The Dynamics of Protein Trafficking and Stability.* 60: 775-781, 1995.

Mostov K, Kaetzel CS. Immunoglobulin transport and the polymeric immunoglobulin receptor. In: Mucosal Immunology. PL Ogra, J Mestecky, M. Lamm, W Strober, J Bienenstock, JR McGhee, editors. San Diego: Academic Press, 1999.

Nahm DH, Kim HY, Park HS. Elevation of specific immunoglobulin A antibodies to both allergen and bacterial antigen in induced sputum from asthmatics. *Eur. Respir. J.* 12:540-5, 1998.

Nahm DH, Park HS. Correlation between IgA antibody and eosinophil cationic protein levels in induced sputum from asthmatic patients. *Clin. Exp. Allergy* 27:676-81, 1997.

Schmidt D, Rabe KF. Immune mechanisms of smooth muscle hyperreactivity in asthma. *J. Allergy Clin. Immunol.* 105:673-82, 2000.

Stewart WW, Mazengera RL, Shen L, Kerr MA. Unaggregated serum IgA binds to neutrophil Fc∀R at physiological concentrations and is endocytosed but cross-linking is necessary to elicit a respiratory burst. *J Leukocyte Biol.* 56481-487, 1994.

WE CLAIM:

1. A method of modulating intracellular calcium signalling in an airway smooth muscle cell comprising administering to a cell or animal in need thereof an effective amount of an agent that can modulate an IgA receptor in an airway smooth muscle cell.

2. A method of preventing or inhibiting intracellular calcium signalling in an airway smooth muscle cell comprising administering to a cell or animal in need thereof an effective amount of an IgA receptor antagonist.

3. A method of inhibiting the contraction of an airway smooth muscle cell comprising administering an effective amount of an IgA receptor antagonist to a cell or animal in need thereof.

4. A method of inhibiting the production of inflammatory mediators or growth factors comprising administering an effective amount of an IgA receptor antagonist to an airway smooth muscle cell or animal in need thereof.

5. A method of treating a condition wherein it is desirable to inhibit a calcium dependent effect in an airway smooth muscle cell comprising administering an effective amount of an IgA receptor antagonist to an animal in need thereof.

6. A method according to claim 5 wherein the condition is asthma, chronic bronchitis, acute bronchitis, bronchial hyperreactivity, chronic obstructive pulmonary disease, emphysema, interstitial lung disease, bronchiectasis or airway remodelling.

7. A method of treating a patient with asthma comprising administering an effective amount of an IgA receptor antagonist to a patient in need thereof.

8. A method according to any one of claims 1 to 7 wherein the IgA receptor is pIgR or FcαR.

9. A method according to any one of claims 2 to 8 wherein the IgA receptor antagonist inhibits the binding of pIgA or pIgM to pIgR.

10. A method according to any one of claims 2 to 8 wherein the IgA receptor antagonist inhibits the binding of any IgA molecule to FcαR.

11. A method according to any one of claims 2 to 10 wherein the IgA receptor antagonist is a scFv that binds pIgR or FcαR.

12. A method of identifying substances which can bind to pIgR on an airway smooth muscle cell comprising the steps of:

(a) reacting pIgR and a test substance, under conditions which allow for formation of a complex between the pIgR and the test substance, and

(b) assaying for complexes of pIgR and the test substance, for free substance or for non complexed pIgR, wherein the presence of complexes indicates that the test substance is capable of binding pIgR.

13. A method of identifying substances which can bind to FcαR on an airway smooth muscle cell comprising the steps of:

(a) reacting FcαR and a test substance, under conditions which allow for formation of a complex between the FcαR and the test substance, and

(b) assaying for complexes of FcαR and the test substance, for free substance or for non complexed FcαR, wherein the presence of complexes indicates that the test substance is capable of binding FcαR.

14. A method of delivering a substance to an airway smooth muscle cell comprising administering an effective amount of a conjugate comprising the

substance coupled to an IgA receptor ligand to an animal or cell in need thereof.

15. A method according to claim 14 wherein the IgA receptor is pIgR or FcαR.

16. A method of detecting a condition associated with the activation of an IgA receptor on an airway smooth muscle cell comprising assaying a sample for (a) a nucleic acid molecule encoding an IgA receptor or a fragment thereof or (b) an IgA receptor or a fragment thereof.

17. A method according to claim 16 wherein the IgA receptor is pIgR or FcαR.

18. A method according to claim 16 or 17 wherein the condition is asthma, chronic bronchitis, acute bronchitis, bronchial hyperreactivity, chronic obstructive pulmonary disease, emphysema, interstitial lung disease, bronchiectasis or airway remodelling.

19. A method of detecting IgA mediated bronchial hyperreactivity comprising:

(a) administering an IgA receptor agonist to a patient; and

(b) detecting bronchoconstriction in the patient wherein an increase in bronchoconstriction as compared to a control indicates that the patient has IgA-mediated hyperreactivity.

20. A method according to claim 19 wherein bronchoconstriction is measured by listening for wheezing on chest auscultation.

21. A method according to claim 19 wherein bronchoconstriction is measured by measuring a reduced forced expiratory volume at 1 second (FEV1).

22. A method of detecting IgA-mediated bronchial hyperreactivity comprising:

(a) administering an IgA-receptor agonist to a patient and detecting bronchoconstriction; and

(b) administering an IgA receptor agonist followed by a non-specific bronchoconstricting agent to the patient and detecting bronchoconstriction at a lower dose than when the nonspecific agent is administered alone wherein bronchoconstriction in step (a) and/or bronchoconstriction induced at a lower dose of the nonspecific agent administered without the IgA receptor agonist in step (b) would indicate that the patient has IgA-mediated bronchial hyperreactivity.

23. A method according to claim 22 wherein the non-specific bronchoconstricting agent is methacholine or histamine.

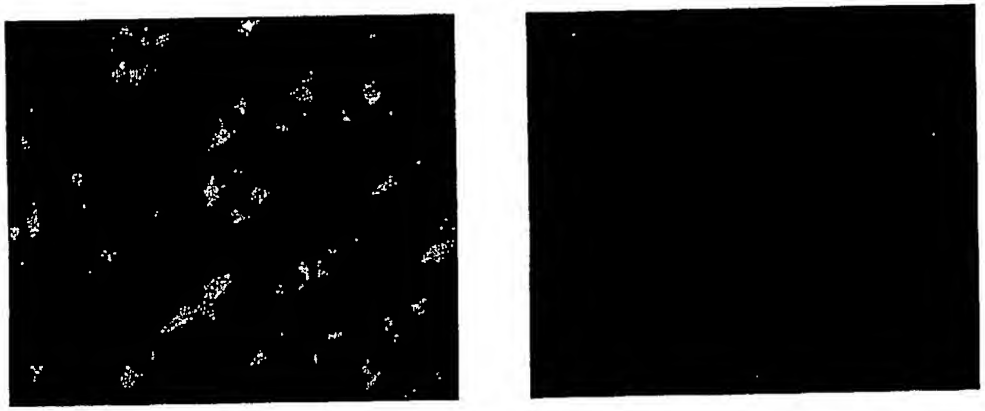
24. A method according to claim 22 or 23 wherein bronchoconstriction is detected with a pulmonary function test such as clinical spirometry [=measurement of FEV1 and FVC].

ABSTRACT OF THE DISCLOSURE

A polymeric immunoglobulin receptor (pIgR) and FcαR have been found on airway smooth muscle (ASM) cell cultures. Incubation of ASM with a ligand for pIgR causes calcium signalling. The invention relates to methods of modulating intracellular calcium signalling in airway smooth muscle cells, methods of treating asthma and methods of drug delivery to airway smooth muscle cells.

Figure 1.A - hpIgR expression in human ASM

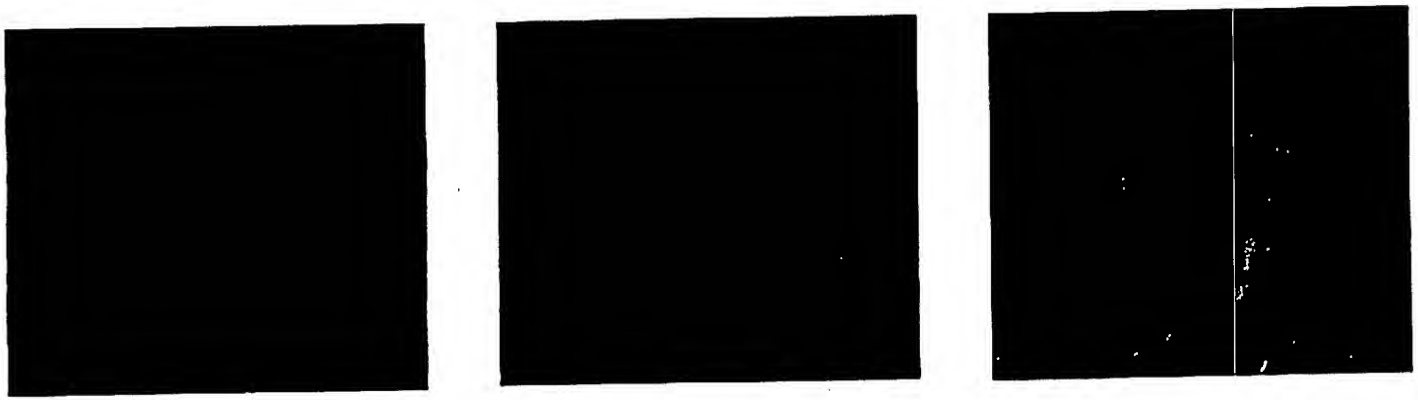
Figure 1.B - Live cell uptake of pIgA by serum-starved ASM



pIgA (0.12 μg/ml)

mIgA (12 μg/ml)

Figure 1.C - Live cell uptake of antibody to pIgR by serum-starved ASM



Serum-free media

Irrelevant rabbit IgG

Rabbit antibody to SC

Figure 2 - FcαR expression in non-serum starved ASM

Pre-incubated with:

- Media

- pIgA (1.2μg/ml)

- mIgA (12μg/ml)

Human ASM were pre-incubated with media alone, pIgA (1.2 μg/ml) or mIgA (12 μg/ml), before fixation and staining with mouse anti FcαR (CD89) and FITC-donkey anti-mouse IgG.

Figure 3 – Western blot for pIgR in ASM

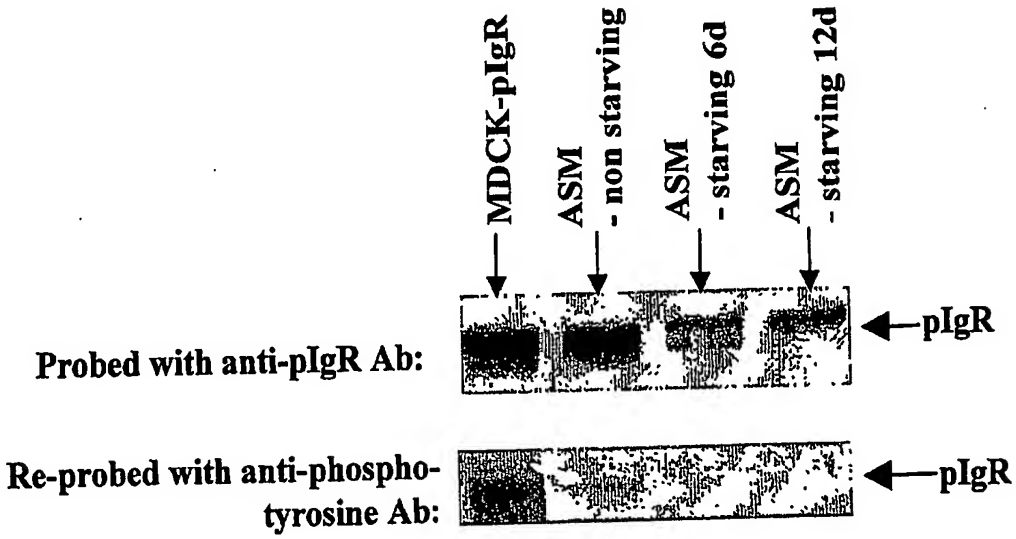


Figure 4 – RT-PCR for pIgR

RNA extracted from serum-starved ASM and HpIgR cells:

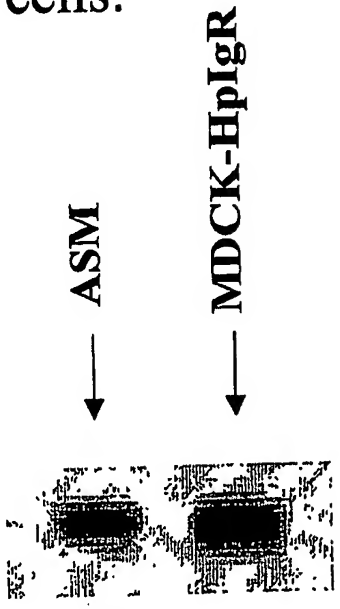


Figure 5 - RT-PCR for FcαR

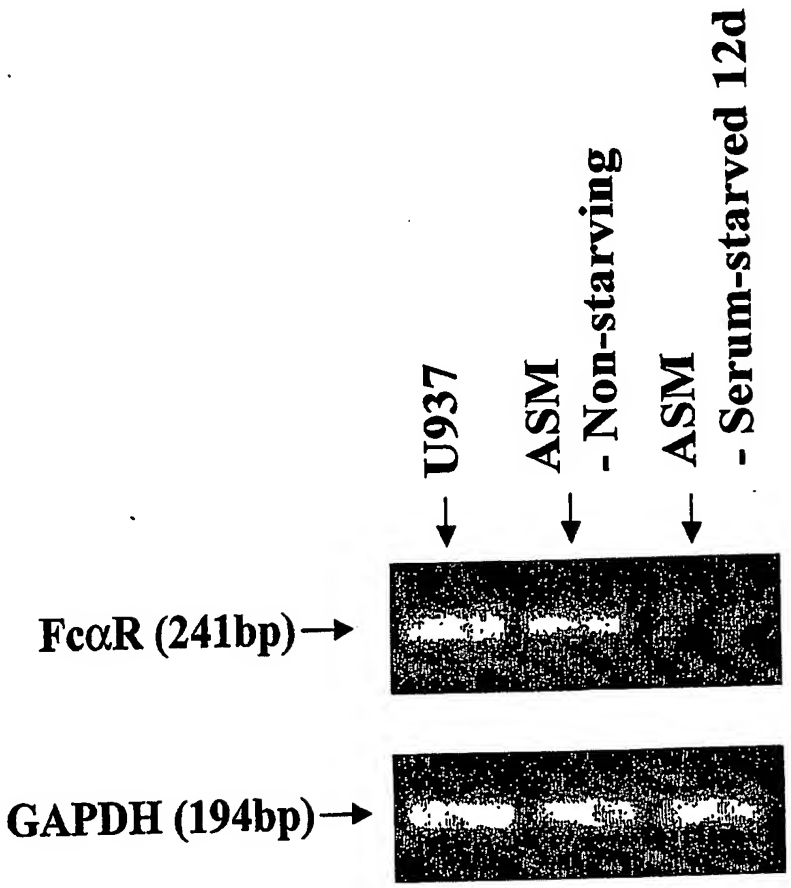


Figure 6A – Fura-2-loaded serum-starved ASM with pIgA (12 μg/ml)

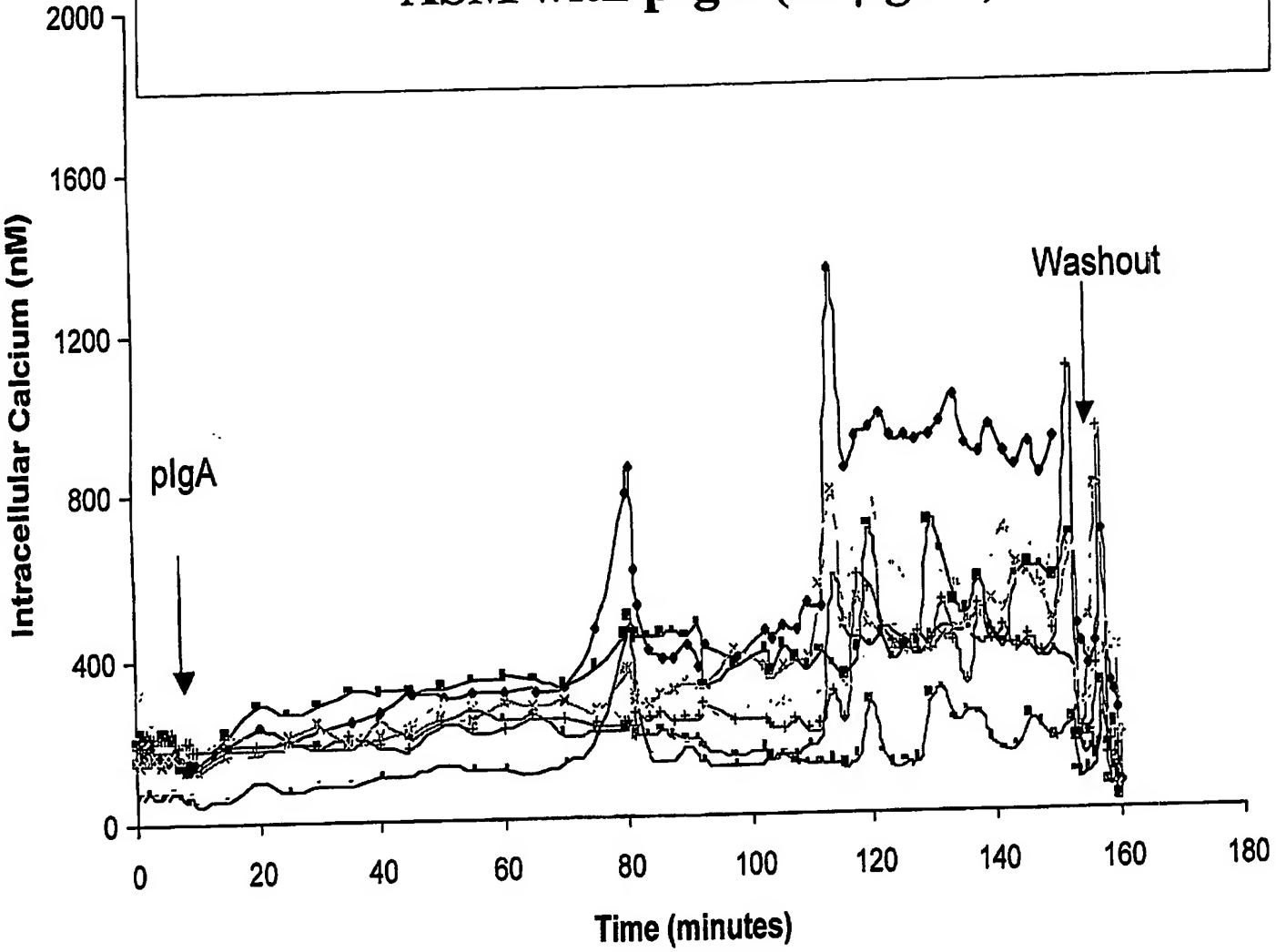


Figure 6B – Fura-2-loaded serum-starved ASM with buffer alone

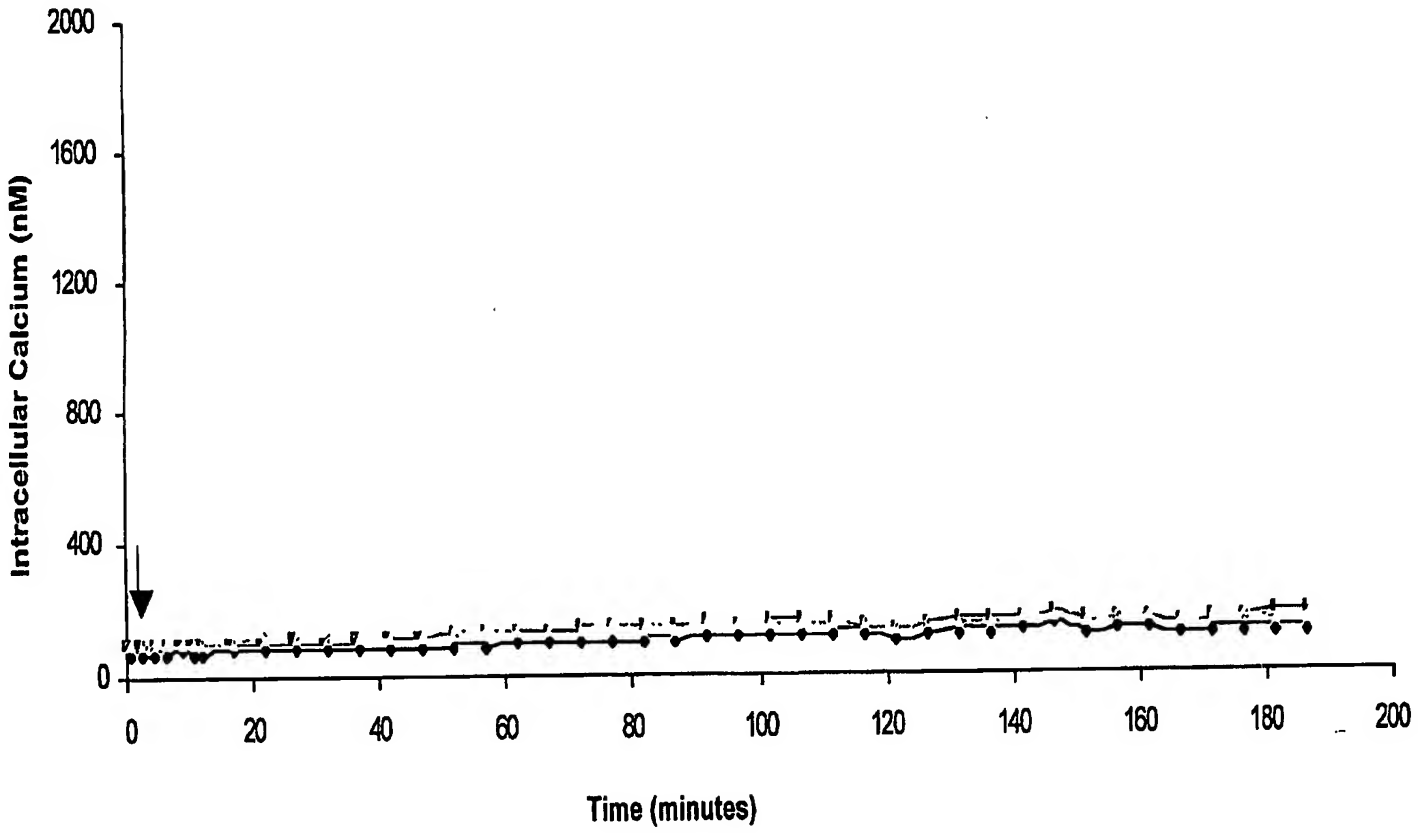


Figure 7A – Histamine and low-dose pIgA

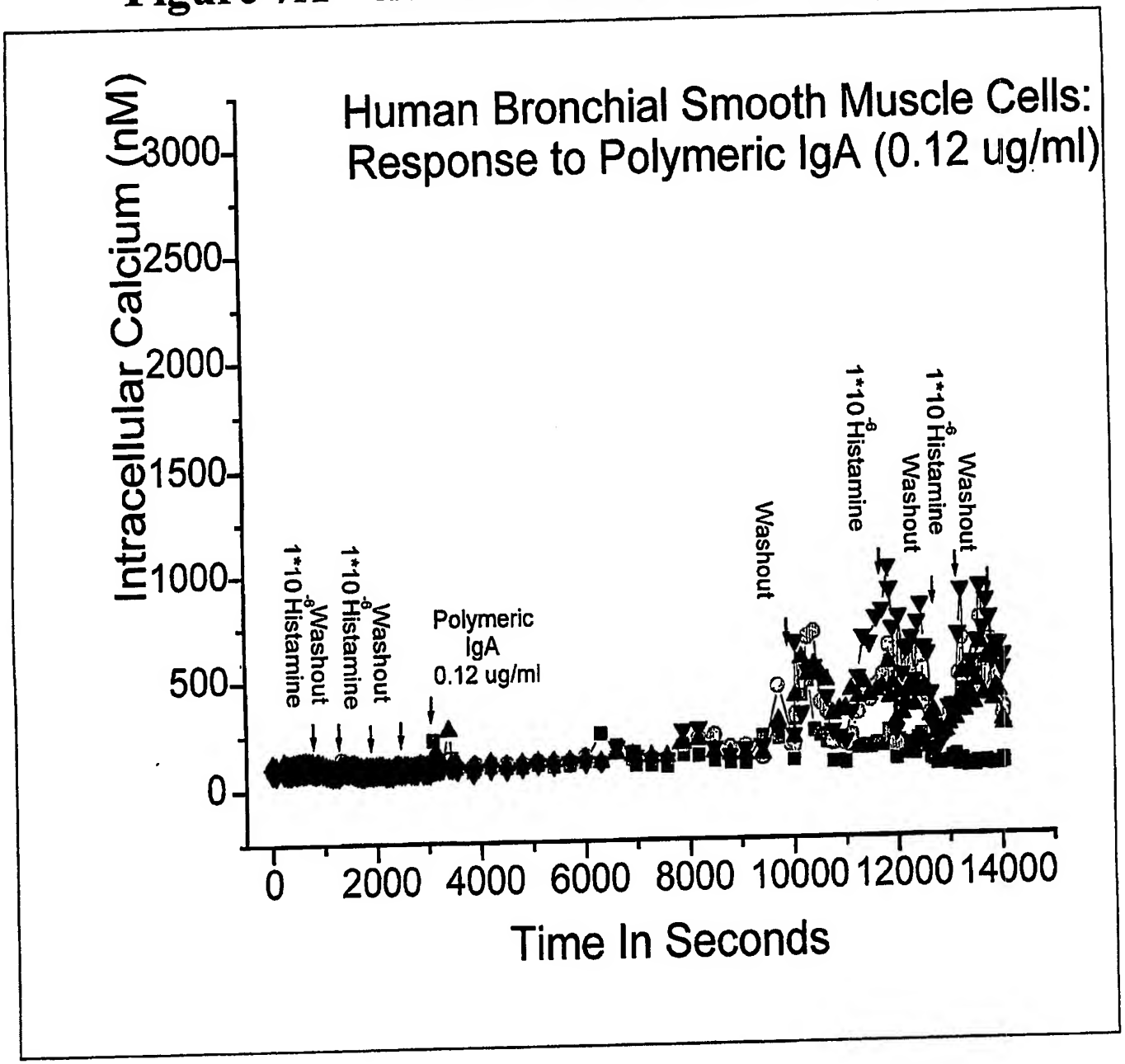


Figure 7B – Histamine and buffer alone

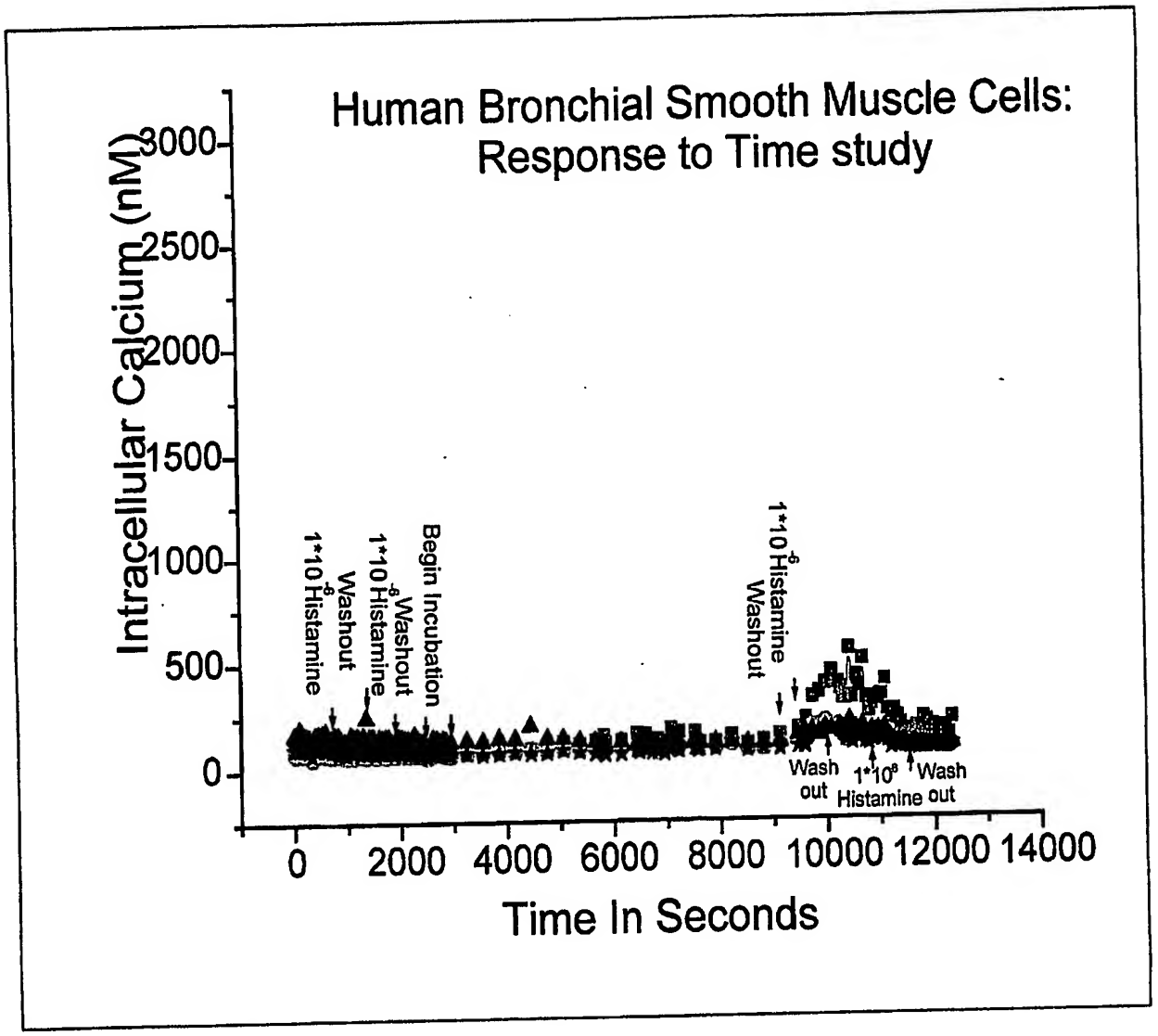


Figure 7C – Histamine and mIgA

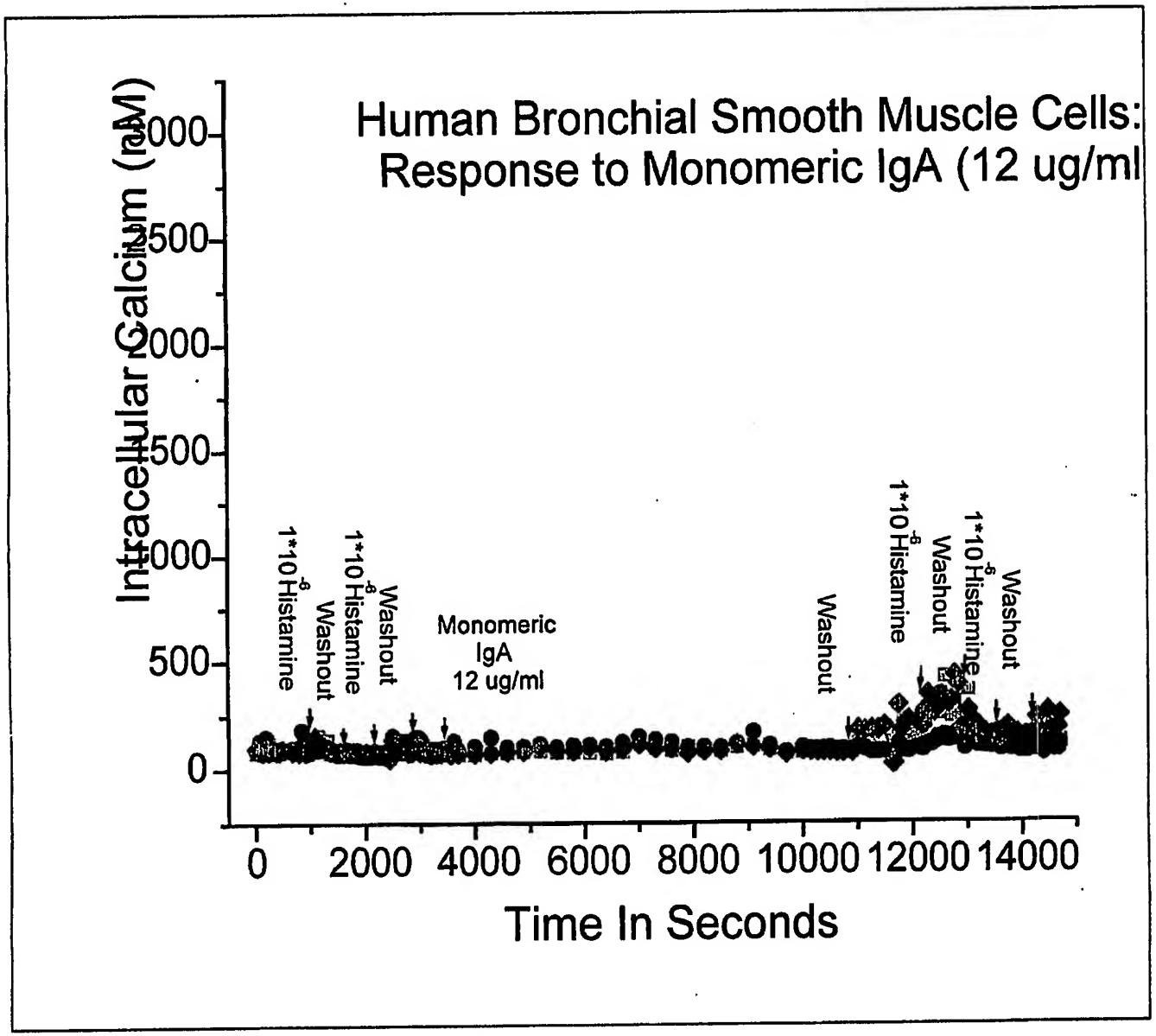


Figure 7D – Histamine and high-dose pIgA

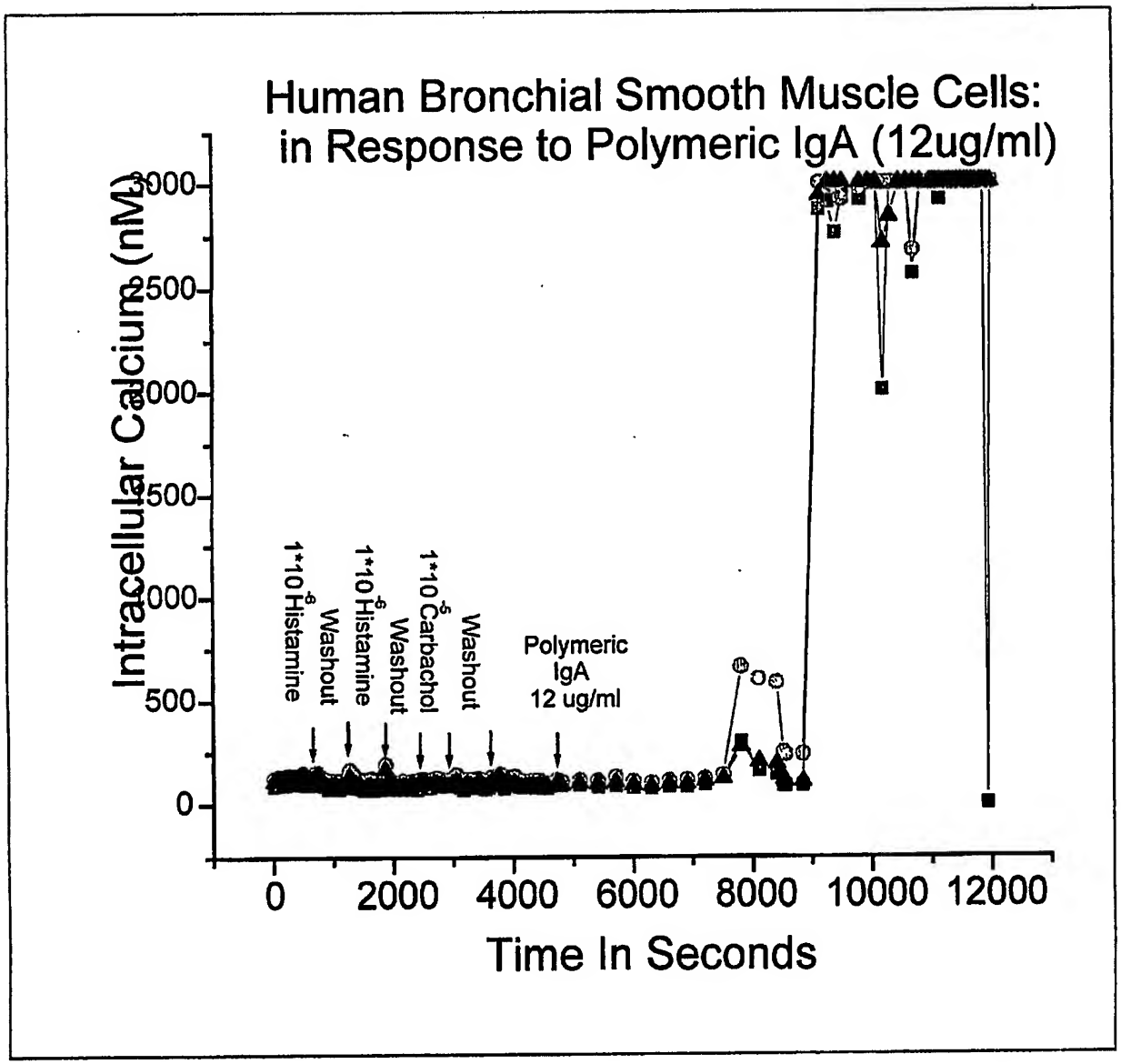


Figure 8 -
ASM + pIgA
12μg/ml
Serial frames
5-10 min apart

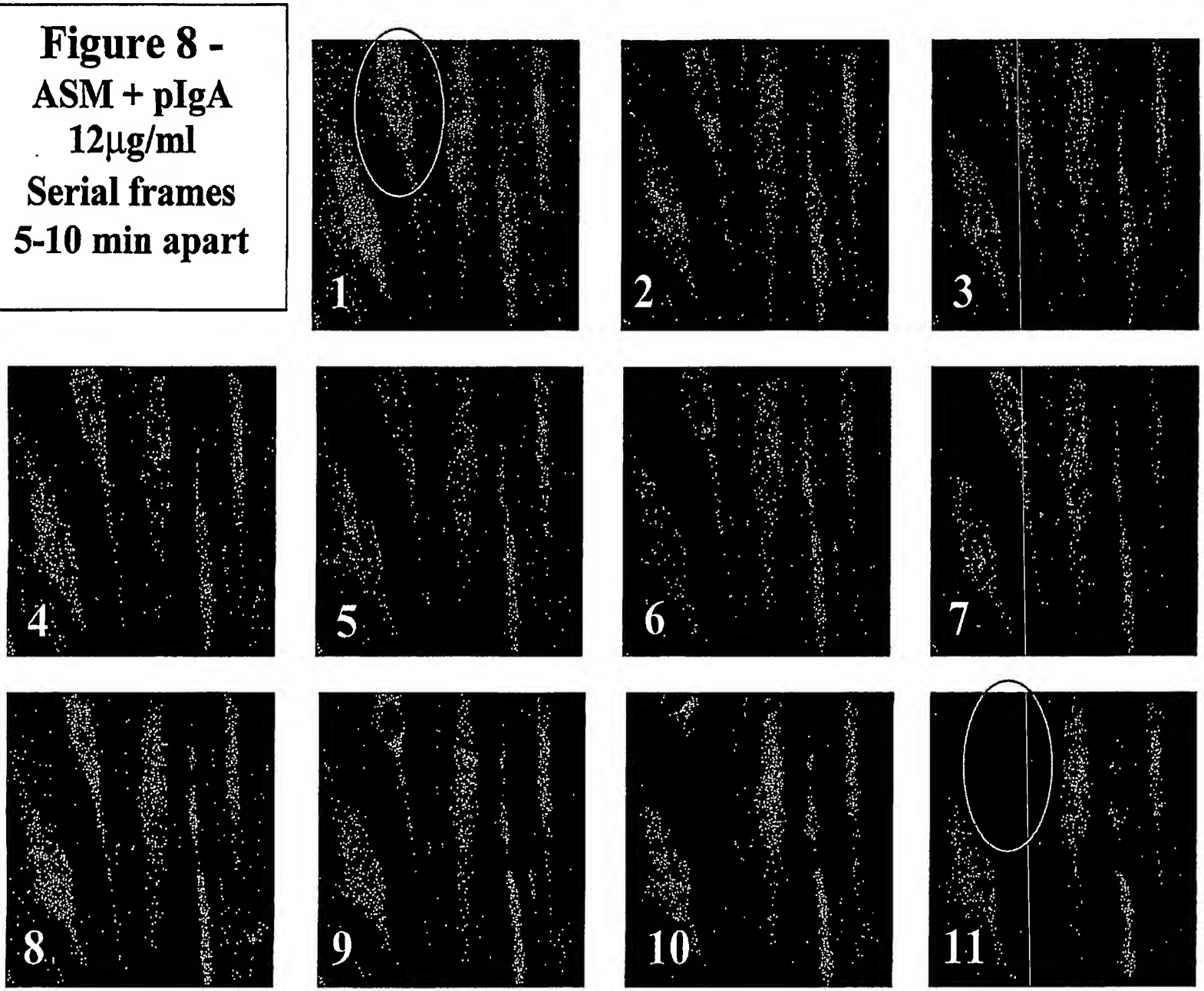


Figure 9: Western blots of candidate scFv binders to J-chain